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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

1,4-Disubstituted-5-hydroxy-3-methylpyrazoles and some derived ring systems as cytotoxic and DNA binding agents. Synthesis, in vitro biological evaluation and in silico ADME study

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Abstract Some novel polysubstituted pyrazoles, bipyrazoles and pyranopyrazoles, supported with various chemotherapeutically-active pharmacophores, were synthesized and biologically evaluated for their cytotoxic potential. Fifteen compounds (**7–9**, **12**, **16**, **17**, **19**, **21**, **22**, **26**, **28**, **30**, **32**, **33**, and **34**) exhibited variable degrees of cytotoxic activity against a panel of three cancer cell lines, among which the analogs **16**, **17**, **21**, **26**, and **34** showed a considerable broad spectrum cytotoxic potential, with special effectiveness against the colon HT29 and breast MCF7 cancer cell lines. In particular, compounds **16**, **17**, and **26** displayed double the activity of doxorubicin against colon carcinoma HT29 cell line, while the pyranopyrazole analog **34** was nearly equiactive with the reference cytotoxic agent. Meanwhile, the analogs **16** and **17** were nearly equipotent to doxorubicin against breast MCF7 cell line. DNA-binding activities of the most active compounds were in agreement with the obtained anticancer activity, where compounds **16**, **17**, **26**, and **34** displayed the highest affinity. In silico calculations of molecular properties revealed that most of the active compounds comply with Lipinski's RO5 and Veber's criteria for good bioavailability suggesting good drug-likeness properties upon oral administration.

Keywords Synthesis · Pyrazoles · Bipyrazoles · Cytotoxicity · DNA binding · Molecular properties

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Introduction

Cancer is a global health concern being one of the most serious causes of illness and early death. World Health Organization (WHO) has estimated more than 13 million deaths from cancer by 2030 (WHO 2016). In particular, breast cancer, colon cancer and hepatocellular carcinoma are amongst the most prominent causes of cancer mortality worldwide. Approximately 230,000 women around the world are diagnosed with breast cancer annually, leading to about 40,000 women death cases. Moreover, one million patients are diagnosed with colon cancer worldwide, where nearly half of them die every year (Siegel et al. 2016). On the other hand, around 500,000 new cases suffering from hepatocellular carcinoma (HCC) are diagnosed each year, where about 85% of which are reported in developing countries, including Egypt. Hepatitis B or C virus infections and alcohol consumption are the main risk aspects for developing HCC (Chatterjee and Mitra 2015). Although cancer chemotherapy has emerged as a major therapeutic discipline, currently available chemotherapeutic drugs face several limitations e.g. normal cells toxicity and cellular drug resistance (Kibria et al. 2014). Therefore, there is an instant demand for the design and discovery of new chemotherapeutic agents, especially for the treatment of the above-mentioned types of cancer.

Among the famous mechanisms standing behind the activity of many chemotherapeutic agents, stemmed the non-covalent interaction with DNA by major/minor groove binders and intercalators as an important mechanism (Thurston 1999). Typical synthetic groove-binding drugs are usually small crescent-shaped molecules that interact with the DNA bases in the minor or major groove of the DNA double helix via multiple hydrogen bonds and Van der Waals interactions. Unlike groove binders, the structure

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of DNA intercalators frequently comprises a planer or semi-planer pharmacophore that enables their insertion between the base pairs of DNA, resulting in conformational changes in the double helix of DNA, with subsequent interruption of replication, transcription, and repair, leading finally to tumor cell death (Hurley 2002; Martinez and Chacon-Garcia 2005). Although the structure of most DNA intercalators contains fused bi/tricyclic ring structures, some atypical intercalators with non-fused ring systems (e.g. prodigiosin) were also reported (Palchaudhuri and Hergenrother 2007). Moreover, many polyfunctionalized small molecules, binuclear and bis compounds were reported to directly interact with DNA in diverse and even complex manners (Paul and Bhattacharya 2012; Wang et al. 2016). Consequently, discovery of small anticancer molecules with DNA-binding capacity is still an active field of research (Arcangeli et al. 2009).

Over the past few years, simple and condensed pyrazole ring systems have attracted considerable interest owing to their diverse chemotherapeutic potential including anti-neoplastic activities, being employed as anticancer (Shi et al. 2015), antileukemic (Manetti et al. 2008), antitumor (Ghorab et al. 2012), cytotoxic (Xu et al. 2013) and anti-proliferative (Cankara Pirol et al. 2014) agents. Among the anticancer pyrazoles, some polysubstituted bipyrazoles have been reported to possess a potential broad spectrum anti-tumor activity in a nanomolar concentration range in the (NCI) in vitro disease-oriented human cells screening panel assay (Rostom 2010). Meanwhile, several pyrazoles have revealed remarkable anticancer potential due to the inhibition of some vital key enzymes necessary for malignant cell division, generation of reactive oxygen species (ROS) (Strocchi et al. 2012; Luo et al. 2014) or DNA-binding activity (Chobe et al. 2012).

On the other hand, several reports have described the anticancer potential of α,β-unsaturated ketones which were reported to function via different mechanisms, including antiproliferative and cytotoxic effects (Szliszka et al. 2010; Orlikova et al. 2012). In particular, small molecules containing arylaminoprop-2-en-1-ones were known to display remarkable cytotoxic and anti-tubulin activity (Reddy et al. 2012; Srinivasa Reddy et al. 2016). Moreover, arylureas and arylamides were found to be embedded in many anticancer drugs with excellent bioavailability and low toxicity (Gamal El-Din et al. 2015). For instance, Sorafenib (having arylurea terminal) has been approved by the U.S. FDA for treating advanced renal cancer (Wilhelm et al. 2006), whereas, Imatinib (possessing an arylamide terminal) proved to be effective in treating chronic myeloid leukemia (CML) with diminished side effects (Capdeville et al. 2002). Additionally, azomethines were known to exhibit stupendous biocidal activities, including anti-proliferative properties (Mishra et al. 2013).

In view of the above-mentioned findings, and in continuation of our efforts devoted to the discovery of novel lead structures as potent chemotherapeutic agents (Rida et al. 2005, 2007; Rostom et al. 2011, 2014; Ashour et al. 2011; Youssef et al. 2012; Faidallah et al. 2015), it was aimed to synthesize and investigate the cytotoxic and DNA-binding potential of some polysubstituted pyrazoles and bipyrazoles with the general structures a–c (Fig. 1). The targeted compounds were designed so as to reserve the hydroxy function at the main pyrazole-C_5 which would act as H-bond donor. Besides, in a trial to assist binding of the aimed compounds with vital molecular targets in the tumor cells, the pyrazole or bipyrazole rings were supplied with various functionalities that would act as H-bond-forming centers e.g. carbonyl, α, β-unsaturated ketone, amino, imino, (thio)ureido, (thio)amide groups hoping to enhance the expected cytotoxic potential. Additionally, the aryl substituent at the main pyrazole-N_1 was selected aiming to confer variable electronic and steric environment which might assist the expected bioactivities. Furthermore, owing to the reported anticancer and DNA-binding activities of many fused pyrans (Bhavanarushi et al. 2013; Gao et al. 2015), annulation of the pyrazole into a pyranopyrazole heterocycle (d; Fig. 1) was considered as an interesting structure modification which might influence the anticipated bioactivities, based on the reported ability of some planner pyrans to bind to DNA through insertion and stacking between the DNA base pairs (Barton 1986). The in vitro cytotoxic potential of the target compounds was investigated against three human cancer cell lines namely; Caucasian breast adenocarcinoma MCF7, hepatocellular carcinoma Hep-G2 and colon carcinoma HT29. The most active compounds were further investigated for their DNA-binding capability as a possible anticancer mechanism. An in silico computation of molecular properties of the biologically active compounds was also performed in order to predict their pharmacokinetic properties (ADME) and their suitability to be developed as oral drug candidates.

Materials and methods

Chemistry

All reagents and solvents were purchased from commercial suppliers and were dried and purified when necessary by standard techniques. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. The infrared (IR) spectra were recorded on Shimadzu FT-IR 8400S infrared spectrophotometer using the KBr pellet technique. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker WM-600 FT NMR spectrometer using tetramethylsilane as the internal standard and DMSO-d_6 as a

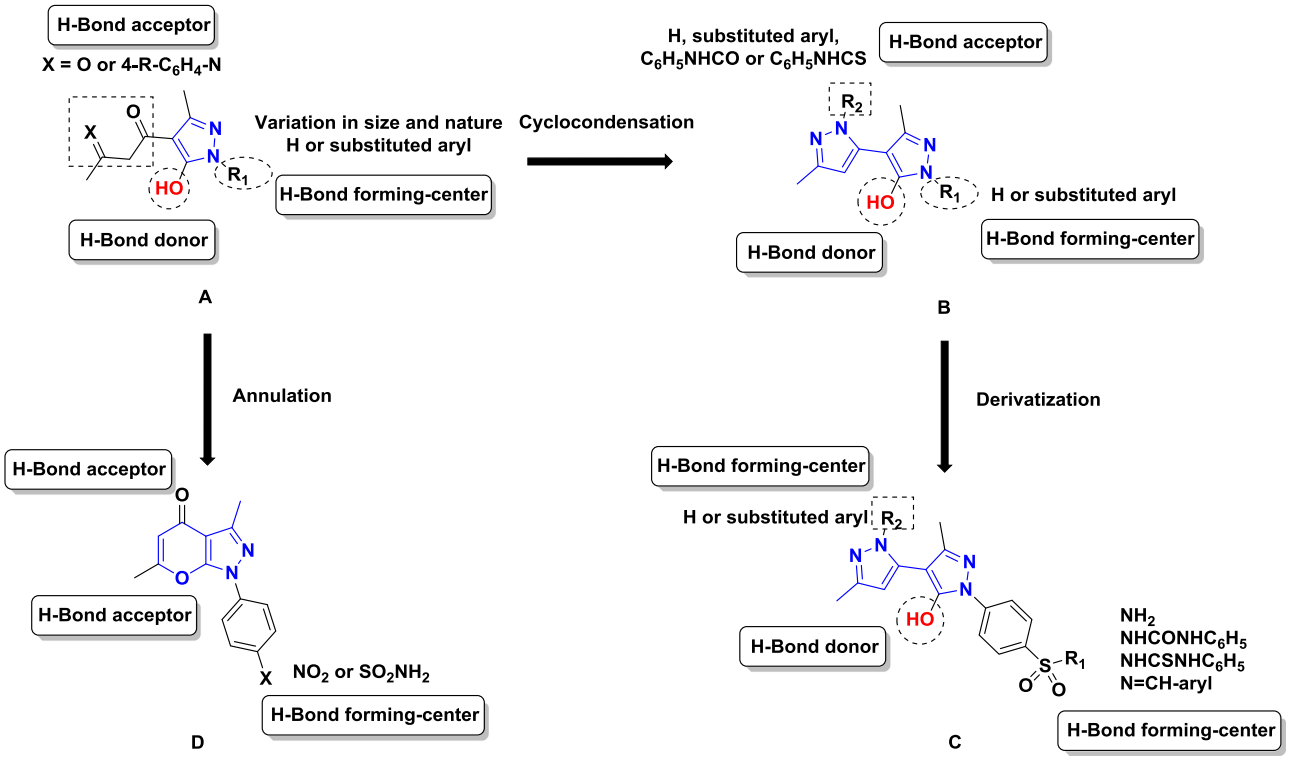


Fig. 1 Structures of the target compounds **a–d**

solvent (Chemical shifts in δ, ppm). Splitting patterns were designated as follows: *s*: singlet; *d*: doublet; *m*: multiplet; *q*: quartet. Elemental analyses were performed on a 2400 Perkin Elmer Series 2 analyzer and the found values were within ±0.4% of the theoretical values. Follow up of the reactions and checking the homogeneity of the compounds were made by TLC on silica gel-protected aluminum sheets (Type 60 F254, Merck) and the spots were detected by exposure to UV-lamp at λ 254. Compounds **2** and **6** (Bendaas et al. 1999), **3**, **7**, **18**, and **20** (Gelin et al. 1983), **12** and **13** (Bendaas et al. 1999; Benaamane et al. 2008) are previously reported and prepared according to the corresponding literature methods.

General procedure for the synthesis of compounds ***2–5***

To a solution of dehydroacetic acid **1** (1.68 g, 10 mmol) in benzene (20 mL), hydrazine hydrate 98% or the appropriate phenyl hydrazine (10 mmol) was added. The reaction mixture was refluxed for 15–30 min then allowed to cool to room temperature for 2 h. The formed precipitate was filtered, washed with benzene and recrystallized from ethanol.

4-Hydroxy-6-methyl-3-(1-(2-(4-nitrophenyl)hydrazono) ethyl)-2*H*-pyran-2-one (**4**) It was obtained as an orange solid; yield: 77%; m.p.: 110–112 °C; IR (KBr, cm^{-1}) υ: 3428, 3293 (OH, NH), 1707 (C=O); ^{1}H NMR (DMSO-d_6) *δ*: 2.47 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 5.43 (s, 1H, pyran-C_5-H), 6.72–7.95 (m, 5H, Ar–H and NH), 16.21 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) *δ*: 16.8 (CH_3), 20.5 (CH_3), 96.8 (Ar-C), 103.5 (Ar–C), 116.1 (Ar–C), 124.5 (Ar–C), 138.5 (Ar–C), 144.4 (Ar–C), 152.8 (Ar–C), 170.4 (Ar–C), 167.8 (C=N), 179.9 (C=O); Anal. Calcd for $C_{14}H_{13}N_3O_5$ (303.27): C, 55.45; H, 4.32; N, 13.86. Found: C, 55.18; H, 4.60; N, 13.62.

4-(2-(1-(4-Hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl) ethylidene)hydrazinyl)benzene-sulfonamide (**5**) It was obtained as a pale yellow solid; yield: 79%; m.p.: 221–223 °C; IR (KBr, cm^{-1}) υ: 3445–3323 (OH, NH, NH_2), 1705 (C=O), 1357, 1140 (SO_2N); ^{1}H NMR (DMSO-d_6) *δ*: 2.46 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 5.63 (s, 1H, pyran-C_5-H), 6.63–8.13 (m, 7H, Ar–H, NH and NH_2), 16.48 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) *δ*: 17.5 (CH_3), 20.2 (CH_3), 96.5 (Ar–C), 103.6 (Ar–C), 115.7 (Ar–C), 126.3 (Ar–C), 129.4 (Ar–C), 141.4 (Ar–C), 149.9 (Ar–C), 174.2 (Ar–C), 161.9 (C=N), 178.9 (C=O); Anal. Calcd. for $C_{14}H_{15}N_3O_5S$ (337.35): C, 49.85; H, 4.48; N, 12.46. Found: C, 49.59; H, 4.24; N, 12.66.

General procedure for the synthesis of compounds ***6–9***

A solution of the appropriate hydrazono derivative **2–5** (10 mmol) in acetic acid (20 mL) was heated under reflux for 1–2 h. The reaction mixture was concentrated, cooled and

the formed precipitate was filtered, washed with cold water, dried and recrystallized from methanol.

1-(5-Hydroxy-3-methyl-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl)butane-1,3-dione (**8**) It was obtained as an orange solid; yield: 81%; m.p.: 187–189 °C; IR (KBr, cm^{-1}) υ: 3415 (OH), 1718, 1632 (C=O); ^{1}H NMR (DMSO-d_6) δ: keto and enolic forms 1.91 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 3.82 (s, 2H, CH_2), 5.24 (s, 1H, CH=), 7.11–7.65 (m, 4H, Ar–H), 14.42 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: keto and enolic forms 15.2 (CH_3), 15.5 (CH_3), 21.6 (CH_3), 30.9 (CH_3), 55.2 (CH_2), 96.4 (CH=), 100.3 (Ar–C), 120.9 (Ar–C), 121.2 (Ar–C), 126.5 (Ar–C), 126.5 (Ar–C), 129.4 (Ar–C), 129.5 (Ar–C), 137.1 (Ar–C), 147.5 (Ar–C), 158.8 (Ar–C), 181.6 (C=O), 188.4 (C=O); Anal. Calcd. for $C_{14}H_{13}N_3O_5$ (303.27): C, 55.45; H, 4.32; N, 13.86. Found: C, 55.54; H, 4.60; N, 13.65.

4-(5-Hydroxy-3-methyl-4-(3-oxobutanoyl)-1*H*-pyrazol-1-yl)benzenesulfonamide (**9**) It was obtained as a pale yellow solid; yield: 85%; m.p.: 214–216 °C; IR (KBr, cm^{-1}) υ: 3420, 3357 (OH, NH_2), 1722, 1620 (C=O), 1360, 1149 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: keto and enolic forms 2.15 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 3.97 (s, 2H, CH_2), 5.21(s, 1H, CH=), 7.64–7.84 (m, 6H, Ar–H and NH_2), 15.28 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: keto and enolic forms 15.6 (CH_3), 16.0 (CH_3), 22.5 (CH_3), 30.2 (CH_3), 54.8 (CH_2), 96.6 (CH=), 100.4 (Ar–C), 120.9 (Ar–C), 121.6 (Ar–C), 126.7 (Ar–C), 126.4 (Ar–C), 129.4 (Ar–C), 129.9 (Ar–C), 137.2 (Ar–C), 147.7 (Ar–C), 157.9 (Ar–C), 180.4 (C=O), 188.3 (C=O); Anal. Calcd. for $C_{14}H_{15}N_3O_5S$ (337.36): C, 49.85; H, 4.48; N, 12.46. Found: C, 49.65; H, 4.42; N, 12.59.

*General procedure for the synthesis of compounds **10–17***

A mixture of **6–9** (10 mmol) and arylamine (10 mmol) in ethanol (20 mL) was heated under reflux for 2 h. After cooling, the obtained solid was filtered, washed with cold ethanol and recrystallized from ethanol.

3-((4-Chlorophenyl)amino)-1-(5-hydroxy-3-methyl-1*H*-pyrazol-4-yl)but-2-en-1-one (**10**) It was obtained a yellow solid; yield: 85%; m.p.: 270–272 °C; IR (KBr, cm^{-1}) υ: 3396, 3293 (OH, NH), 1675 (C=O); ^{1}H NMR (DMSO-d_6) δ: 2.53 (s, 3H, CH_3), 2.58 (s, 3H, CH_3), 5.46 (s, 1H, CH=), 7.22–7.75 (m, 6H, Ar–H and 2NH), 12.27 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.8 (CH_3), 94.4 (CH=), 117.5 (Ar–C), 126.4 (Ar–C), 129.2 (Ar–C), 129.5 (Ar–C), 136.4 (Ar–C), 146.4 (Ar–C), 160.0 (Ar–C), 131.9 (HN–C=), 184.7 (C=O); Anal. Calcd. for $C_{14}H_{14}ClN_3O_2$ (291.74): C, 57.64; H, 4.84; N, 14.40. Found: C, 57.42; H, 4.88; N, 14.25.

1-(5-Hydroxy-3-methyl-1*H*-pyrazol-4-yl)-3-(p-tolylamino)but-2-en-1-one (**11**) It was obtained as a pale yellow solid; yield: 92%; m.p.: 263–264 °C; IR (KBr, cm^{-1}) υ: 3388, 3299 (OH, NH), 1678 (C=O); ^{1}H NMR (DMSO-d_6) δ: 2.15 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 5.48 (s, 1H, CH=), 7.10–7.78 (m, 6H, Ar–H and 2NH), 12.38 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.5 (CH_3), 21.4 (CH_3), 94.7 (CH=), 117.5 (Ar–C), 126.3 (Ar–C), 129.6 (Ar–C), 129.4 (Ar–C), 136.4 (Ar–C), 146.3 (Ar–C), 160.0 (Ar–C), 131.9 (HN–C=), 184.7 (C=O); Anal. Calcd. for $C_{15}H_{17}N_3O_2$ (271.32): C, 66.40; H, 6.32; N, 15.49. Found: C, 66.60; H, 6.26; N, 15.72.

3-((4-Chlorophenyl)amino)-1-(5-hydroxy-3-methyl-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl)but-2-en-1-one (**14**) It was obtained as a yellow solid; yield: 91%; m.p.: 202–204 °C; IR (KBr, cm^{-1}) υ: 3400, 3268 (OH, NH), 1670 (C=O); ^{1}H NMR (DMSO-d_6) δ: 2.32 (s, 3H, CH_3), 2.63 (s, 3H, CH_3), 5.18 (s, 1H, CH=), 7.28–7.56 (m, 9H, Ar–H and NH), 12.39 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.4 (CH_3), 20.4 (CH_3), 94.6 (CH=), 100.8 (Ar–C), 117.5 (Ar–C), 120.3 (Ar–C), 126.6 (Ar–C), 129.4 (Ar–C), 129.7 (Ar–C), 136.7 (Ar–C), 137.4 (Ar–C), 146.2 (Ar–C), 160.5 (Ar–C), 161.4 (Ar–C), 131.9 (HN–C=), 184.9 (C=O); Anal. Calcd. for $C_{20}H_{17}ClN_4O_4$ (412.83): C, 58.19; H, 4.15; N, 13.57. Found: C, 58.25; H, 4.18; N, 13.82.

1-(5-Hydroxy-3-methyl-1-(4-nitrophenyl)-1*H*-pyrazol-4-yl)-3-(p-tolylamino)but-2-en-1-one (**15**) It was obtained as an orange solid; yield: 88%; m.p.: 192–194 °C; IR (KBr, cm^{-1}) υ: 3397, 3271 (OH, NH), 1669 (C=O); ^{1}H NMR (DMSO-d_6) δ: 2.34 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 2.85 (s, 3H, CH_3), 5.43 (s, 1H, CH=), 7.09–7.54 (m, 9H, Ar–H and NH), 12.11 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.7 (CH_3), 20.6 (CH_3), 21.5 (CH_3), 94.5 (CH=), 100.5 (Ar–C), 117.3 (Ar–C), 120.7 (Ar–C), 126.2 (Ar–C), 129.2 (Ar–C), 129.3 (Ar–C), 136.8 (Ar–C), 137.7 (Ar–C), 146.4 (Ar–C), 159.9 (Ar–C), 161.2 (Ar–C), 131.6 (HN–C=), 184.6 (C=O); Anal. Calcd. for $C_{21}H_{20}N_4O_4$ (392.42): C, 64.28; H, 5.14; N, 14.28. Found: C, 64.14; H, 5.41; N, 14.38.

4-(4-(3-((4-Bromophenyl)amino)but-2-enoyl)-5-hydroxy-3-methyl-1*H*-pyrazol-1-yl)benzenesulfonamide (**16**) It was obtained as a yellow solid; yield: 92%; m.p.: 323–325 °C; IR (KBr, cm^{-1}) υ: 3402–3268 (OH, NH, NH_2), 1673 (C=O), 1372, 1153 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.33 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 5.65 (s, 1H, CH=), 7.18–7.79 (m, 11H, Ar–H, NH and NH_2), 12.56 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.8 (CH_3), 94.4 (CH=), 100.8 (Ar–C), 117.3 (Ar–C), 120.5 (Ar–C), 126.6 (Ar–C), 129.4 (Ar–C), 129.6 (Ar–C), 136.9 (Ar–C), 137.9 (Ar–C), 146.6 (Ar–C), 160.5 (Ar–C), 161.3 (Ar–C), 131.2 (HN-C=), 184.5 (C=O);

Anal. Calcd. for $C_{20}H_{19}BrN_4O_4S$ (491.37): C, 48.89; H, 3.90; N, 11.40. Found: C, 48.65; H, 3.88; N, 11.32.

4-(4-(3-((4-Fluorophenyl)amino)but-2-enoyl)-5-hydroxy-3-methyl-1*H*-pyrazol-1-yl)benzenesulfonamide (**17**) It was obtained as a yellow solid; yield: 92%; m.p.: 138–140 °C; IR (KBr, cm^{-1}) υ: 3416–3208 (OH, NH, NH_2), 1674 (C=O), 1363, 1151 (SO_2N); ^1H NMR (DMSO-d_6) δ: 2.32 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 5.27 (s, 1H, CH=), 7.23–7.82 (m, 11H, Ar–H, NH and NH_2), 12.28 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.9 (CH_3), 20.7 (CH_3), 94.6 (CH=) 100.9 (Ar–C), 117.4 (Ar–C), 120.3 (Ar–C), 126.4 (Ar–C), 129.5 (Ar–C), 129.2 (Ar–C), 136.8 (Ar–C), 137.6 (Ar–C), 146.5 (Ar–C), 160.8 (Ar–C), 161.6 (Ar–C), 131.9 (HN–C=), 184.7 (C=O); Anal. Calcd. for $C_{20}H_{19}FN_4O_4S$ (430.46): C, 55.81; H, 4.45; N, 13.02. Found: C, 55.86; H, 4.62; N, 13.18.

*General procedure for the synthesis of bipyrazole derivatives **18–24***

A solution of **7–9** (10 mmol) and hydrazine hydrate 98% or the appropriate phenyl hydrazine (10 mmol) in ethanol (20 mL) was heated under reflux for 2–3 h. The reaction mixture was cooled and the formed precipitate was filtered, washed with cold ethanol, dried and recrystallized from ethanol.

4-(5′-Hydroxy-3′,5-dimethyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)benzenesulfonamide (**19**) It was obtained as a pale yellow solid; yield: 70%; m.p.: 285–287 °C; IR (KBr, cm^{-1}) υ: 3454–3387 (OH, NH, NH_2), 1368, 1148 (SO_2N); ^1H NMR (DMSO-d_6) δ: 1.95 (s, 3H, CH_3), 2.63 (s, 3H, CH_3), 6.82 (s, 1H, pyrazole-C_4-H), 7.23–7.73 (m, 6H, Ar–H and NH_2), 8.85 (s, 1H, NH, D_2O exchangeable), 12.68 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 11.1 (CH_3), 14.3 (CH_3), 95.9 (Ar–C), 101.3 (Ar–C), 112.7 (Ar–C), 120.9 (Ar–C), 125.7 (Ar–C), 128.9 (Ar–C), 138.6 (Ar–C), 140.6 (Ar–C), 145.5 (Ar–C), 146.6 (Ar–C); Anal. Calcd. for $C_{14}H_{15}N_5O_3S$ (333.37): C, 50.44; H, 4.54; N, 21.01. Found: C, 50.32; H, 4.79; N, 21.18.

4-(5′-Hydroxy-3′,5-dimethyl-2-phenyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)benzenesulfonamide (**21**) It was obtained as a pale yellow solid; yield: 75%; m.p.: 311–313 °C; IR (KBr, cm^{-1}) υ: 3387–3298 (OH, NH_2), 1370, 1156 (SO_2N); ^1H NMR (DMSO-d_6) δ: 2.38 (s, 3H, CH_3), 2.79 (s, 3H, CH_3), 6.14 (s, 1H, pyrazole-C_4-H), 7.13–7.69 (m, 11H, Ar–H and NH_2), 13.54 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 11.8 (CH_3), 14.1 (CH_3), 95.8 (Ar–C), 101.4 (Ar–C), 109.7 (Ar–C), 112.7 (Ar–C), 120.3 (Ar–C), 125.5 (Ar–C), 128.8 (Ar–C), 138.6 (Ar–C), 140.40 (Ar–C), 143.3 (Ar–C), 145.5 (Ar–C), 146.6 (Ar–C), 149.3 (Ar–C), 151.3 (Ar–C); Anal. Calcd. for $C_{20}H_{19}N_5O_3S$ (409.47): C, 58.67; H, 4.68; N, 17.10. Found: C, 58.38; H, 4.75; N, 17.18.

4,4′-(5′-Hydroxy-3′,5-dimethyl-1′*H*,2*H*-[3,4′-bipyrazole]-1′,2-diyl)dibenzenesulfonamide (**22**) It was obtained as a pale yellow solid; yield: 78%; m.p.: 334–336 °C; IR (KBr, cm^{-1}) υ: 3381–3287 (OH, NH_2), 1366, 1159 (SO_2N); ^1H NMR (DMSO-d_6) δ: 2.58 (s, 3H, CH_3), 2.85 (s, 3H, CH_3), 6.45 (s, 1H, pyrazole-C_4-H), 7.22–7.73 (m, 12H, Ar–H and NH_2), 11.74 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 11.5 (CH_3), 14.6 (CH_3), 95.8 (Ar–C), 101.6 (Ar–C), 112.2 (Ar–C), 120.3 (Ar–C), 125.3 (Ar–C), 128.7 (Ar–C), 138.4 (Ar–C), 140.6 (Ar–C), 145.4 (Ar–C), 146.3 (Ar–C); Anal. Calcd. for $C_{20}H_{20}N_6O_5S_2$ (488.54): C, 49.17; H, 4.13; N, 17.20. Found: C, 49.06; H, 4.35; N, 17.28.

3′,5-Dimethyl-1′-(4-nitrophenyl)-2-phenyl-1′*H*,2*H*-[3,4′-bipyrazol]-5′-ol (**23**) It was obtained as an orange solid; yield: 75%; m.p.: 260–262 °C; IR (KBr, cm^{-1}) υ: 3392 (OH); ^1H NMR (DMSO-d_6) δ: 2.64 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 6.76 (s, 1H, pyrazole-C_4-H), 7.31–7.92 (m, 9H, Ar–H), 12.27 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 11.6 (CH_3), 14.5 (CH_3), 95.6 (Ar–C), 101.3 (Ar–C), 112.2 (Ar–C), 118.4 (Ar–C), 120.9 (Ar–C), 125.4 (Ar–C), 128.9 (Ar–C), 138.4 (Ar–C), 140.6 (Ar–C), 143.6 (Ar–C), 145.4 (Ar–C), 146.8 (Ar–C), 149.2 (Ar–C), 151.7 (Ar–C); Anal. Calcd. for $C_{20}H_{17}N_5O_3$ (375.39): C, 63.99; H, 4.56; N, 18.66. Found: C, 63.73; H, 4.75; N, 18.92.

3′,5-Dimethyl-1′,2-bis(4-nitrophenyl)-1′*H*,2*H*-[3,4′-bipyrazol]-5′-ol (**24**) It was obtained as an orange solid; yield: 77%; m.p.: 184–186 °C; IR (KBr, cm^{-1}) υ: 3380 (OH); ^1H NMR (DMSO-d_6) δ: 2.53 (s, 3H, CH_3), 2.66 (s, 3H, CH_3), 6.19 (s, 1H, pyrazole-C_4-H), 7.27–8.10 (m, 8H, Ar–H), 13.07 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 11.7 (CH_3), 14.5 (CH_3), 95.3 (Ar–C), 101.1 (Ar–C), 112.6 (Ar–C), 120.4 (Ar–C), 125.6 (Ar–C), 128.7 (Ar–C), 138.4 (Ar–C), 140.5 (Ar–C), 145.5 (Ar–C), 146.5 (Ar–C); Anal. Calcd. for $C_{20}H_{16}N_6O_5$ (420.39): C, 57.14; H, 3.84; N, 19.99. Found: C, 57.18; H, 3.58; N, 19.84.

*General procedure for the synthesis of compounds **25–28***

To a stirred mixture of the appropriate bipyrazole **18** or **21** (10 mmol) and anhydrous K_2CO_3 (1.4 g, 10 mmol) in dry acetone (25 mL), a solution of phenyl isocyanate or phenyl isothiocyanate (10 mmol) in dry acetone (5 mL) was added drop wise. The reaction mixture was heated under reflux for 8–10 h. The solvent was removed under reduced pressure and the remaining solid residue was dissolved in water (30 mL) and neutralized with 2 N HCl. The obtained solid product was filtered, washed with water, dried and recrystallized from dimethylformamide.

5′-Hydroxy-3′,5-dimethyl-*N*,1′-diphenyl-1′*H*,2*H*-[3,4′-bipyrazole]-2-carboxamide (**25**) It was obtained as a pale yellow solid; yield: 65%; m.p.: 233–235 °C; IR (KBr, cm^{-1}) υ: 3382, 3313 (OH, NH), 1646 (C=O); ^1H NMR

(DMSO-d_6) δ: 2.18 (s, 3H, CH_3), 2.52 (s, 3H, CH_3), 6.32 (s, 1H, pyrazole-C_4-H), 6.89–7.88 (m, 11H, Ar–H and NH), 11.74 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.3 (CH_3), 97.2 (Ar–C), 101.7 (Ar–C), 114.5 (Ar–C), 115.3 (Ar–C), 123.8 (Ar–C), 123.9 (Ar–C), 130.1 (Ar–C), 130.9 (Ar–C), 138.7 (Ar–C), 143.2 (Ar–C), 150.8 (Ar–C), 160.4 (Ar–C), 162.1 (Ar–C), 165.2 (Ar–C), 173.8 (C=O); Anal. Calcd. for $C_{21}H_{19}N_5O_2$ (373.42): C, 67.55; H, 5.13; N, 18.76. Found: C, 67.71; H, 5.21; N, 18.68.

5′-Hydroxy-3′,5-dimethyl-*N*,1′-diphenyl-1′*H*,2*H*-[3,4′-bipyrazole]-2-carbothioamide (**26**) It was obtained as a pale yellow solid; yield: 66%; m.p.: 245–247 °C; IR (KBr, cm^{-1}) υ: 3375, 3332 (OH, NH), 1158 (C=S); ^{1}H NMR (DMSO-d_6) δ: 2.11 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 6.38 (s, 1H, pyrazole-C_4-H), 6.72–7.41 (m, 10H, Ar–H), 8.22 (s, 1H, NH, D_2O exchangeable), 14.76 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.2 (CH_3), 20.5 (CH_3), 105.9 (Ar–C), 110.5 (Ar–C), 118.9 (Ar–C), 120.5 (Ar–C), 124.1 (Ar–C), 126.0 (Ar–C), 128.8 (Ar–C), 129.0 (Ar–C), 129.1 (Ar–C), 136.3 (Ar–C), 138.2 (Ar–C), 139.7 (Ar–C), 144.4 (Ar–C), 154.2 (Ar–C), 163.4 (C=S); Anal. Calcd. for $C_{21}H_{19}N_5OS$ (389.48): C, 64.76; H, 4.92; N, 17.98. Found: C, 64.64; H, 5.03; N, 17.87.

4-(5′-Hydroxy-3′,5-dimethyl-2-phenyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)-*N*-(phenylcarbamoyl)benzenesulfonamide (**27**) It was obtained as a pale yellow solid; yield: 68%; m.p.: 257–259 °C; IR (KBr, cm^{-1}) υ: 3354–3267 (OH, NH), 1658 (C=O), 1368, 1182 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.35 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 6.73 (s, 1H, pyrazole-C_4-H), 7.06–7.82 (m, 16H, Ar–H and 2NH), 12.14 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.4 (CH_3), 20.3 (CH_3), 97.4 (Ar–C), 101.3 (Ar–C), 101.6 (Ar–C), 114.2 (Ar–C), 123.9 (Ar–C), 123.9 (Ar–C), 130.0 (Ar–C), 130.5 (Ar–C), 138.9 (Ar–C), 143.2 (Ar–C), 147.7 (Ar–C), 149.3 (Ar–C), 153.4 (Ar–C), 160.6 (Ar–C), 161.7 (Ar–C), 162.3 (Ar–C), 165.8 (Ar–C), 169.4 (Ar–C), 173.5 (C=O); Anal. Calcd. for $C_{27}H_{24}N_6O_4S$ (528.59): C, 61.35; H, 4.58; N, 15.90. Found: C, 61.62; H, 4.25; N, 15.82.

4-(5′-Hydroxy-3′,5-dimethyl-2-phenyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)-*N*-(phenylcarbamothioyl)benzenesulfonamide (**28**) It was obtained as a pale yellow solid; yield: 69%; m.p.: 286–288 °C; IR (KBr, cm^{-1}) υ: 3363–3288 (OH, NH), 1348, 1174 (SO_2N), 1160 (C=S); ^{1}H NMR (DMSO-d_6) δ: 2.52 (s, 3H, CH_3), 2.86 (s, 3H, CH_3), 6.93 (s, 1H, pyrazole-C_4-H), 7.08–7.82 (m, 16H, Ar–H and 2NH), 11.92 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.0 (CH_3), 21.2 (CH_3), 94.4 (Ar–C), 101.3 (Ar–C), 112.3 (Ar–C), 114.6 (Ar–C), 120.1 (Ar–C), 120.5 (Ar–C), 122.9 (Ar–C), 123.2 (Ar–C), 123.5 (Ar–C), 126.4 (Ar–C), 130.1 (Ar–C), 130.5 (Ar–C), 131.3 (Ar–C), 143.8 (Ar–C), 147.7 (Ar–C), 153.4 (Ar–C), 160.5 (Ar–C), 160.8 (Ar–C), 184.6 (C=S); Anal. Calcd. for $C_{27}H_{24}N_6O_3S_2$ (544.66): C, 59.54; H, 4.44; N, 15.43. Found: C, 59.51; H, 4.23; N, 15.18.

General procedure for the synthesis of compounds ***29–32***

A mixture of the bipyrazole **19** or **21** (10 mmol) and the appropriate aldehyde (10 mmol) in acetic acid (25 mL) was heated under reflux for 8 h. The reaction mixture was concentrated and left to cool to room temperature. The separated solid was filtered, washed with ethanol and recrystallized from dimethylformamide.

N-(4-Bromobenzylidene)-4-(5′-hydroxy-3′,5-dimethyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)benzenesulfonamide (**29**) It was obtained as a yellow solid; yield: 76%; m.p.: 244–246 °C; IR (KBr, cm^{-1}) υ: 3362, 3290 (OH, NH), 1332, 1150 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.16 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 6.69 (s, 1H, pyrazole-C_4-H), 7.22–7.65 (m, 9H, Ar–H and NH), 8.18 (s, 1H, CH=N), 15.53 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.9 (CH_3), 94.4 (Ar–C), 100.9 (Ar–C), 113.3 (Ar–C), 117.4 (Ar–C), 120.2 (Ar–C), 122.4 (Ar–C), 126.1 (Ar–C), 128.6 (Ar–C), 129.5 (Ar–C), 130.6 (Ar–C), 137.4 (Ar–C), 139.5 (Ar–C), 146.2 (Ar–C), 147.4 (Ar–C), 161.4 (CH=N); Anal. Calcd. for $C_{21}H_{18}BrN_5O_3S$ (500.38): C, 50.41; H, 3.63; N, 14.00. Found: C, 50.21; H, 3.52; N, 14.25.

N-(2,4-Difluorobenzylidene)-4-(5′-hydroxy-3',5-dimethyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)benzenesulfonamide (**30**) It was obtained as an orange solid; yield: 68%; m.p.: 262–264 °C; IR (KBr, cm^{-1}) υ: 3348, 3312 (OH, NH), 1342, 1155 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.64 (s, 3H, CH_3), 2.95 (s, 3H, CH_3), 6.11 (s, 1H, pyrazole-C_4-H), 7.28–7.59 (m, 8H, Ar–H and NH), 8.23 (s, 1H, CH=N), 15.14 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.3 (CH_3), 94.5 (Ar–C), 100.9 (Ar–C), 113.7 (Ar–C), 117.9 (Ar–C), 120.8 (Ar–C), 122.9 (Ar–C), 126.5 (Ar–C), 128.8 (Ar–C), 129.6 (Ar–C), 130.8 (Ar–C), 137.2 (Ar–C), 139.5 (Ar–C), 146.6 (Ar–C), 147.6 (Ar–C), 158.9 (Ar–C), 160.3 (Ar–C), 161.5 (CH=N); Anal. Calcd. for $C_{21}H_{17}F_2N_5O_3S$ (457.46): C, 55.14; H, 3.75; N, 15.31. Found: C, 55.26; H, 3.77; N, 15.18.

N-Benzylidene-4-(5′-hydroxy-3′,5-dimethyl-2-phenyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)benzenesulfonamide (**31**) It was obtained as a pale yellow solid; yield: 70%; m.p.: 306–308 °C; IR (KBr, cm^{-1}) υ: 3338 (OH), 1336, 1156 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.36 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 6.46 (s, 1H, pyrazole-C_4-H), 7.23–7.57 (m, 14H, Ar–H), 8.19 (s, 1H, CH=N), 15.46 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.8 (CH_3), 20.7 (CH_3), 94.8 (Ar–C), 100.9 (Ar–C), 113.2 (Ar–C), 117.6 (Ar–C), 120.6 (Ar–C), 122.6 (Ar–C), 126.3 (Ar–C), 127.5 (Ar–C), 128.7 (Ar–C), 129.3 (Ar–C), 129.3 (Ar–C), 130.5 (Ar–C), 137.8 (Ar–C), 139.9 (Ar–C), 146.7 (Ar–C), 147.3 (Ar–C), 158.7 (Ar–C), 160.3 (Ar–C), 161.2 (CH=N); Anal.

Calcd. for $C_{27}H_{23}N_5O_3S$ (497.57): C, 65.18; H, 4.66; N, 14.08. Found: C, 65.35; H, 4.41; N, 14.16.

N-(4-Bromobenzylidene)-4-(5′-hydroxy-3′,5-dimethyl-2-phenyl-1′*H*,2*H*-[3,4′-bipyrazol]-1′-yl)benzenesulfonamide (**32**) It was obtained as a yellow solid; yield: 72%; m.p.: 246–248 °C; IR (KBr, cm^{-1}) υ: 3352 (OH), 1345, 1166 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.20 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 6.28 (s, 1H, pyrazole-C_4-H), 7.21–7.67 (m, 13H, Ar–H), 8.20 (s, 1H, CH=N), 15.23 (s, 1H, OH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ: 15.9 (CH_3), 20.8 (CH_3), 94.9 (Ar–C), 100.9 (Ar–C), 113.6 (Ar–C), 117.8 (Ar–C), 120.6 (Ar–C), 122.8 (Ar–C), 126.1 (Ar–C), 127.6 (Ar–C), 128.9 (Ar–C), 129.1 (Ar–C), 129.1 (Ar–C), 130.5 (Ar–C), 137.3 (Ar–C), 139.9 (Ar–C), 146.5 (Ar–C), 147.1 (Ar–C), 158.7 (Ar–C), 160.1 (Ar–C), 161.0 (CH=N); Anal. Calcd. for $C_{27}H_{22}BrN_5O_3S$ (576.46): C, 56.25; H, 3.85; N, 12.15. Found: C, 56.32; H, 4.02; N, 12.23.

*General procedure for the synthesis of compounds **33** and **34***

To a stirred ice cold solution of the appropriate pyrazole diketone **8** or **9** (10 mmol) in acetic acid (20 mL), concentrated sulfuric acid (1 mL) was added drop wise. The reaction mixture was stirred at room temperature for 2 h, then poured onto ice-water. The formed precipitate was filtered, washed several times with cold water, dried and recrystallized from dimethylformamide.

3,6-Dimethyl-1-(4-nitrophenyl)pyrano[2,3-c]pyrazol-4(1*H*)-one (**33**) It was obtained as an orange solid; yield: 66%; m.p.: 196–198 °C; IR (KBr, cm^{-1}) υ: 1666 (C=O); ^{1}H NMR (DMSO-d_6) δ: 2.12 (s, 3H, CH_3), 2.62 (s, 3H, CH_3), 5.43 (s, 1H, pyranopyrazole-C_5-H), 8.14 (d, $J = 9.2$ Hz, 2H, 4-nitrophenyl-$C_{2,6}$-H), 8.32 (d, $J = .2$ Hz, 2H, 4-nitrophenyl-$C_{3,5}$-H); ^{13}C NMR (DMSO-d_6) δ: 15.7 (CH_3), 21.9 (CH_3), 98.5 (Ar–C), 110.2 (Ar–C), 119.3 (Ar–C), 124.7 (Ar–C), 129.2 (Ar–C), 145.8 (Ar–C), 154.9 (Ar–C), 153.0 (Ar–C), 164.9 (Ar–C), 187.1 (C=O); Anal. Calcd. for $C_{14}H_{11}N_3O_4$ (285.26): C, 58.95; H, 3.89; N, 14.73. Found: C, 58.79; H, 4.02; N, 14.52.

4-(3,6-Dimethyl-4-oxopyrano[2,3-c]pyrazol-1(4*H*)-yl) benzenesulfonamide (**34**) It was obtained as a pale yellow solid; yield: 68%; m.p.: 272–274 °C; IR (KBr, cm^{-1}) υ: 3354, 3149 (NH_2); 1664 (C=O); 1375, 1152 (SO_2N); ^{1}H NMR (DMSO-d_6) δ: 2.04 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 5.32 (s, 1H, pyranopyrazole-C_5-H), 6.89–7.43 (m, 6H, Ar–H and NH_2); ^{13}C NMR (DMSO-d_6) δ: 15.9 (CH_3), 20.7 (CH_3), 93.1 (Ar–C), 114.4 (Ar–C), 120.4 (Ar–C), 126.8 (Ar-C), 128.9 (Ar–C), 131.1 (Ar–C), 138.0 (Ar–C), 146.4 (Ar–C), 162.9 (Ar–C), 183.7 (C=O); Anal. Calcd. for $C_{14}H_{13}N_3O_4S$ (319.34): C, 52.66; H, 4.10; N, 13.16. Found: C, 52.54; H, 4.12; N, 13.23.

Biology

In vitro MTT cytotoxicity assay

The synthesized compounds were investigated for their in vitro cytotoxic effect via the standard [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (MTT) (Mosmann 1983; Denizot and Lang 1986) against a panel of three human tumor cell lines namely; Caucasian breast adenocarcinoma MCF7, hepatocellular carcinoma Hep-G2, and colon carcinoma HT29. The following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Stanford, ME, USA). Cells were batch cultured for 10 days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of the test compounds. DMSO was employed as a vehicle for dissolution of the tested compounds and its final concentration on the cells was less than 0.2%. Cells were suspended in RPMI 1640 medium (for Hep-G2 and HT29 cell lines) and DMEM (for MCF7 cell line), 1% antibiotic-antimycotic mixture (10,000 IU/mL penicillin potassium, 10,000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B), and 1% L-glutamine in 96-well flat bottom microplate at 37 °C under 5% CO_2. After 48 h of incubation, the medium was aspirated, 40 µL of MTT salt (2.5 µg/mL) were added to each well and incubated for further 4 h at 37 °C under 5% CO_2. To stop the reaction and dissolve the formed crystals, 200 µL of 10% sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37 °C. A positive control which is composed of 100 µg/mL of Doxorubicin, a known cytotoxic natural agent was used. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent *t*-test by SPSS 11 program. The results are presented in Table 1 as LC_{50} (µM) which is the lethal concentration of the compound which causes death of 50% of the cells in 24 h.

DNA-binding assay on TLC-plate

Analysis of the DNA binding affinity of the tested compounds was performed using RP-TLC plates (RP-18 F254; 0.25 mm Merck). TLC plates were pre-developed with methanol/water mixture (8:2). Test compounds were

Table 1 Cytotoxic effects (LC_{50}; μM) of the active compounds on three human tumor cell lines using the MTT assay

Compd number	Human tumor cell line		
	Colon carcinoma HT29	Hepatocellular carcinoma Hep-G2	Breast cancer MCF7
7	87.0	78.0	–[a]
8	69.4	41.4	59.8
9	56.3	89.5	–
12	48.9	59.6	83.4
16	20.6	25.4	3.7
17	18.3	26.9	4.2
19	70.7	81.1	29.2
21	46.6	36.1	23.7
22	98.0	54.0	45.5
26	21.5	6.9	13.1
28	62.8	41.7	79.2
30	78.2	46.5	66.4
32	75.6	–	69.5
33	68.9	–	35.9
34	40.3	31.6	7.1
Doxorubicin[b]	38.8	3.1	3.9

LC_{50} Lethal concentration of the compound which causes death of 50% of cells in 24 h (μM)

[a] Totally inactive against this cell line

[b] Positive control cytotoxic agent

spotted (5 mg/mL in methanol) at the base line, followed by introducing DNA (1 mg/mL in water and methanol mixture 8:2) at the same positions at the origin. The plates were then developed with the above-mentioned elution system, and the location of DNA was visualized by spraying with anisaldehyde, which gives a blue color with DNA. Ethidium bromide was utilized as positive control (Pezzuto et al. 1991).

Methyl green-DNA displacement colorimetric assay

DNA-methyl green complex (20 mg) was suspended in 100 mL of 0.05 M TriseHCl buffer, pH 7.5, containing 7.5 mmol $MgSO_4$ and stirred at 37 °C for 24 h. Compounds to be tested were dissolved in EtOH in Eppendorff tubes and the solvent was then removed under reduced pressure, and 200 mL of the DNA-methyl green solution was added to each tube. The initial absorbance of each sample was measured at 630 nm, and the samples were incubated in the dark at room temperature for 24 h. Thereafter, the final absorbance of each sample was measured and the readings were corrected for initial absorbance and normalized as a percentage of the untreated DNA-methyl green absorbance value. Results were recorded as IC_{50} for each compound which is the sample concentration required to produce 50% reduction in the initial absorbance of the DNA-methyl green solution (Burres et al. 1992).

In silico computation of molecular properties (ADME prediction) of the active compounds

A computational study of the active compounds **7–9**, **12**, **16**, **17**, **19**, **21**, **22**, **26**, **28–30**, **32**, and **34** was performed for the prediction of ADME properties. In this study, we have calculated Lipinski's rule of five which includes logarithm of partition coefficient (CLog P), molecular weight (MW), number of hydrogen bond acceptors (HBA) and number of hydrogen bond donors (HBN). Also, number of rotatable bonds (n-ROTB), topological polar surface area (TPSA), and molecular volume (MV) were predicted using Molinspiration online property calculation toolkit (www.molinspiration.com). Absorption (% ABS) was calculated by:

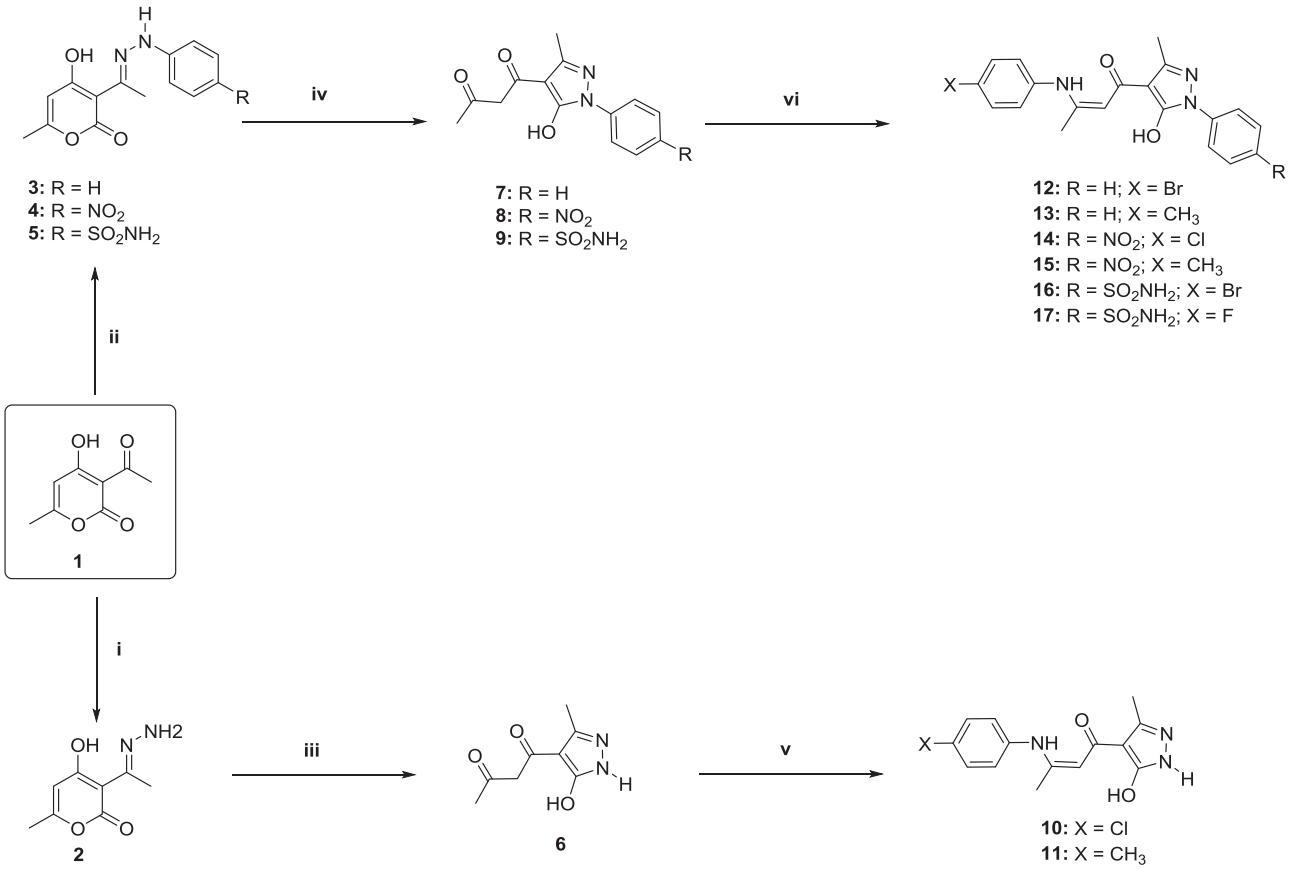
$$\%\,\mathrm{ABS} = 109 - (0.345\,9\,\mathrm{TPSA}).$$

Results and discussion

Chemistry

3-Acetyl-4-hydroxy-6-methyl-2-oxo-2*H*-pyran, which is also known as dehydroacetic acid (DHA), was first discovered by Geuther (1866). It was considered as a versatile starting material for the synthesis of a wide variety of heterocyclic ring systems through ring opening and recyclization (Ait-Baziz et al. 2010; Cantos et al. 1987; Susnik et al. 1992). Consequently, we report in this work the synthesis of different pyrazoles, bipyrazoles and pyranopyrazoles using DHA as a starting material.

The pathways adopted to obtain the intermediates and target compounds are illustrated in Schemes 1 and 2. Scheme 1 starts with hydrazinolysis of the commercially available DHA **1** with hydrazine hydrate or the appropriate aryl hydrazine in benzene to afford the corresponding hydrazono derivatives **2–5**, according to Gelin et al. (1983). Treatment of the latter hydrazones with glacial acetic acid yielded the key intermediates diketopyrazoles **6–9** through a rearrangement reaction. The mechanism behind this reaction is explained in Fig. 2, where a nitrogen nucleophilic attack at the lactone C_2-carbonyl is involved, followed by ring opening to afford the diketopyrazoles **6–9**. Their IR spectra revealed two absorption bands at 1620–1632 and 1718–1722 cm^{-1} characteristic for the two carbonyl groups, in addition to the OH absorption band. The ^{1}H-NMR spectra of compounds **8** and **9** showed that they are present



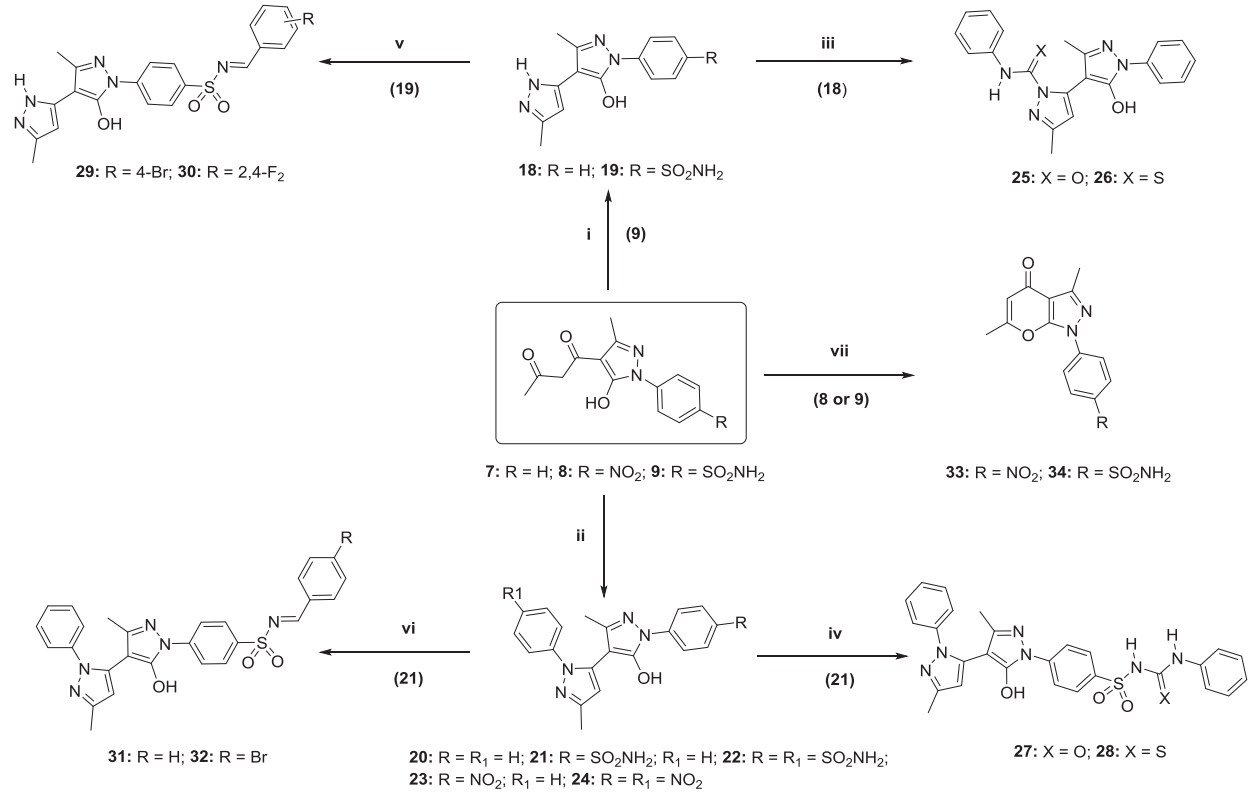
Scheme 1 Synthesis of compounds **2–17**

as an equilibrium mixture between the keto form a and the two enolic forms **b** and **c** (Fig. 3). That was proved by the presence of a singlet at δ 3.82 and 3.97 characteristic for the methylene protons of the keto form and a singlet at δ 5.21 and 5.24 attributed to the vinyl proton of the enol structure. In addition, four singlet signals were assigned to the two methyl protons of the keto form and the two methyl protons of the enol form at their respective chemical shifts. The ^{13}C-NMR spectra revealed two signals at δ 54.8–55.2 and 96.4–96.6 due to the methylene and the vinyl carbons of the keto and the enol forms, respectively, in addition to the signals of the four methyl carbons at their expected chemical shifts.

On the other hand, refluxing **6–9** with the appropriate arylamine in ethanol yielded the target pyrazolylenaminones **10–17**. Their IR spectra lacked one of the two carbonyl absorption bands and characterized by the appearance of a new band at 3208–3299 cm^{-1}due to the NH group. Their ^{1}H-NMR showed a singlet of one proton intensity at δ 5.18–5.65 assigned to the C=CH. Also, the ^{13}C-NMR spectra revealed the =CH signal at δ 94.4–94.7.

Shifting to Scheme 2, the same diketopyrazoles key intermediates **7–9** were used for the synthesis of the target compounds **18–34**. In this context, heating the diketopyrazoles **7–9** with hydrazine hydrate or the appropriate aryl hydrazine in ethanol furnished the corresponding bipyrazoles **18–24**. The compounds were characterised by the disappearance of the carbonyl bands in the IR spectra, whereas their ^{1}H-NMR spectra exhibited a new singlet at δ 6.14–6.82 due to the pyrazole-C_4-H.

Condensation of bipyrazole derivative **18** with either phenyl isocyanate or isothiocyanate in dry acetone containing anhydrous potassium carbonate yielded the corresponding carbamoyl and thiocarbamoyl derivatives **25** and **26**, respectively. Analogously, treating the bipyrazole **21** with phenyl isocyanate or isothiocyanate in dry acetone gave rise to the corresponding substituted ureido and thioureido derivatives **27** and **28**, respectively. The IR spectra of compounds **25** and **27** showed a carbonyl absorption band at 1646 and 1658 cm^{-1}, while compounds **26** and **28** revealed a thiocarbonyl band at 1158 and 1160 cm^{-1}, respectively. Further confirmation of structures arises



Scheme 2 Synthesis of compounds **18–34**

from their ^{13}C NMR spectral data which exhibited a carbonyl carbon signal at δ 173.8 and 173.5 for compounds **25** and **27** and a thiocarbonyl carbon at δ 163.4 and 184.6 for the analogs **26** and **28**, respectively.

Moreover, condensation of the bipyrazole derivatives **19** and **21** with appropriate aromatic aldehyde in acetic acid yielded the corresponding N-arylidenebenzene-sulfonamide derivatives **29–32**. Their IR spectra exhibited two sharp bands at 1332–1345 and 1150–1166 cm^{-1} owing to the sulfonyl moiety. In addition, the compounds were characterized by the appearance of a singlet at δ 8.18–8.23 in the ^1H NMR spectrum due to the arylidene proton. Finally, the pyranopyrazole derivatives **33** and **34** were obtained by treating compounds **8** and **9** with a mixture of acetic acid and sulfuric acid. Their ^1H NMR spectra revealed a singlet at δ 5.43 and 5.32, respectively attributed to the pyranopyrazole-C_5-H.

Biology

In vitro MTT cytotoxicity assay

The synthesized target compounds **6–34** have been investigated for their in vitro cytotoxic potential via the standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Mosmann 1983; Denizot and Lang 1986) against a panel of three human tumor cell lines namely; colon carcinoma HT29, hepatocellular carcinoma Hep-G2 and Caucasian breast adenocarcinoma MCF7. Doxorubicin was employed as a positive control cytotoxic agent. The results are presented in Table 1 as LC_{50} (μM) which is the lethal concentration of the compound that causes death of 50% of the cells in 24 h.

The obtained data revealed that fifteen compounds namely; **7–9**, **12**, **16**, **17**, **19**, **21**, **22**, **26**, **28**, **30**, **32**, **33**, and **34** were able to affect the viability of the tested tumor cell lines particularly the colon HT29 cancer cell line which exhibited a variable degree of sensitivity against all the active compounds. An outstanding cytotoxic activity was displayed by compounds **16**, **17**, and **26** (LC_{50} 20.6, 18.3, and 21.5 μM, respectively), which was nearly two-folds greater than doxorubicin (LC_{50} 38.8 μM). Moreover, a remarkable cytotoxic potential was exhibited by compounds **12**, **21**, and **34** against the same cell line (LC_{50} 48.9, 46.6, and 40.3 μM, respectively), among which the pyranopyrazole analog **34** was nearly equiactive with doxorubicin (LC_{50} 40.3 vs. 38.8 μM, respectively). Whereas, compounds **8**, **9**, **19**, **28**, **30**, **32**, and **33** (LC_{50} range 56.3–78.2 μM)

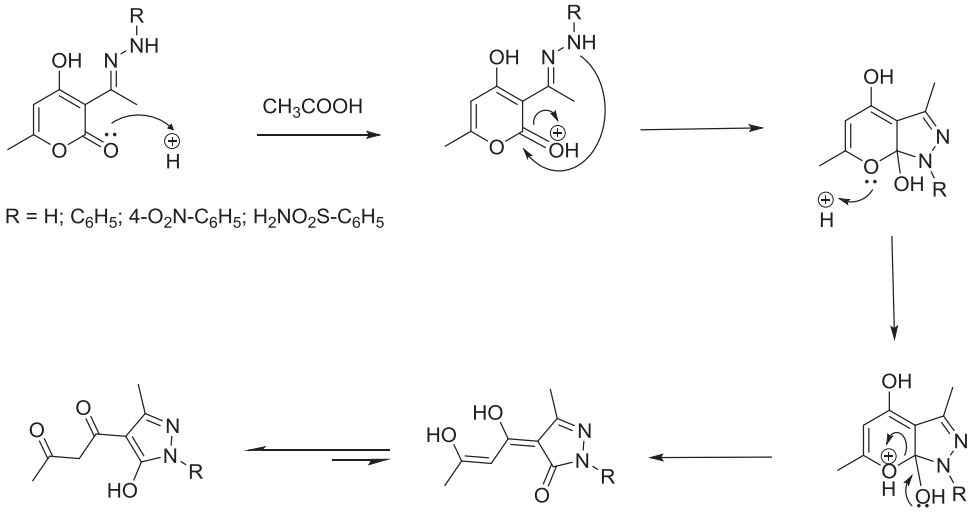


Fig. 2 Reaction mechanism and ring opening phenomenon in the synthesis of compounds **6–9**

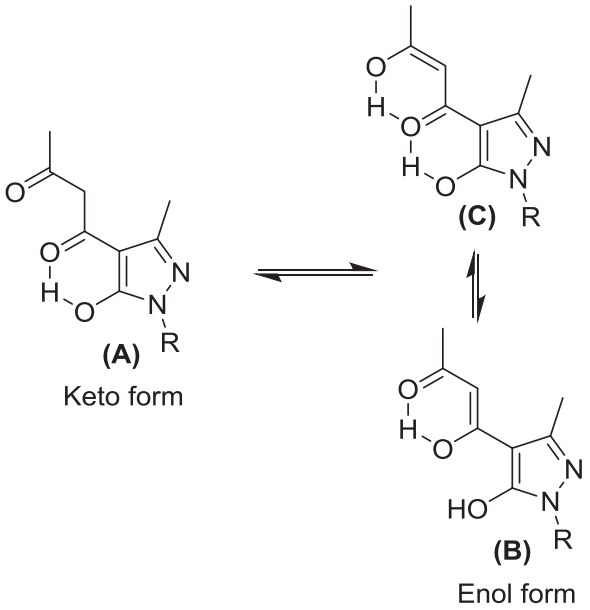


Fig. 3 Tautomerization of compounds **3** and **9–11**

revealed moderate cytotoxic potential against the same cell line, representing nearly 50–70% of activity of doxorubicin. Regarding the growth of breast MCF7 cell line, it was found to be variably inhibited by thirteen of the active compounds with LC_{50} values ranging between 3.7 and 83.4 µM. A significant growth inhibitory potential was displayed by compounds **16** and **17** (LC_{50} 3.7 and 4.2 µM, respectively), which were nearly equipotent to doxorubicin (LC_{50} 3.9 µM). Furthermore, an observable cytotoxic potential was shown by the analogs **26** and **34** (LC_{50} 13.1 and 7.1 µM, respectively) representing about 30 and 55% of the activity of doxorubicin against the same cell line, respectively. On the other hand, the Hep-G2 was proved to be the least sensitive cell cancer line in this study as its growth was moderately to weakly affected by fourteen compounds, with LC_{50} values range 25.4–78.0 µM, except the analog **26** (LC_{50} 6.9 µM) which displayed almost 50% of the activity of doxorubicin (LC_{50} 3.1 µM). Collectively, compounds **16**, **17**, **21**, **26**, and **34** showed a considerable broad spectrum cytotoxic potential against the three tested human tumor cell lines, with special effectiveness against the colon HT29 and breast MCF7 cancer cell lines (Table 1).

Structure-activity correlation

A close examination of the structures of the active compounds revealed that better cell growth inhibitory activity was confined to derivatives encountering an aryl moiety at N_1 of the main 1-aryl-5-hydroxy-3-methyl-1*H*-pyrazole scaffold rather than those with unsubstituted N_1. Moreover, the cytotoxic efficiency seems to be modulated by the nature of the aryl substituent at the N_1 of the pyrazole scaffold. Obviously, better cytotoxic activity could be correlated with derivatives encountering the sulfonamido substituent ($R{=}SO_2NH_2$), while those unsubstituted or with a 4-nitro substituent ($R{=}NO_2$) showed mild or even no activity. In this context, among the diketopyrazoles **6–9**, compound **9** ($R{=}SO_2NH_2$) was the most active member showing about 70% of the activity of doxorubicin against the colon HT29 cell line. Derivatization of the diketopyrazoles **6–9** into the corresponding arylaminobut-2-en-1-ones **10–17** gave rise an obvious enhancement in both cytotoxic potential and spectrum, among which the sulfonamide derivatives **16** and **17** ($R{=}SO_2NH_2$; $R_1{=}$Br or F, respectively) were the most active. Both analogs exhibited about double the activity of doxorubicin against the colon HT29, beside being nearly equipotent with the reference drug against the breast MCF7 cell lines. On the other hand, cycloaddition of the key intermediates **7–9** with different hydrazines as in compounds **18–24** led to a complete abolishment of activity, except the bipyrazole sulfonamide analogs **19**, **21**, and **22**

Table 2 DNA binding affinity and DNA-methyl green displacement assay of compounds **16**, **21**, **17**, **26**, and **34**

Compound	DNA binding affinity	IC_{50} (mmol/mL)[a]
16	High	0.059 ± 0.004
17	High	0.051 ± 0.003
21	Moderate	0.093 ± 0.005
26	High	0.049 ± 0.002
34	High	0.047 ± 0.003
Ethidium bromide	High	0.004 ± 0.006

[a] Values represent the concentration (mean ± SD, $n = 6$ separate determinations) required to cause 50% decrease in the initial absorbance of DNA-methyl green complex

($R=SO_2NH_2$) which showed moderate broad spectrum cytotoxic activity against the three tested cell lines. Prominently, compound **21** was nearly equiactive to doxorubicin against the colon HT29 cell line. Incorporation of a phenylcarbamoyl moiety at N_1 of the new pyrazole ring (**25;** X=O), did not offer a significant advantage to the cytotoxic efficiency. However, bioisosteric replacement of the carbamoyl group with a thiocarbamoyl one as in **26** (X=S), led to a significant enhancement in the overall cytotoxic potency and spectrum of activity against the three tested cell lines. On the other hand, derivatization of the bipyrazole sulfonamide **21** ($R=SO_2NH_2$, $R_1=H$) to the corresponding sulfonylureido (**27**; X=O), sulfonylthioureido (**28;** X=S) and imino (**31**; R=H and **32**; R=Br) derivatives led to a noticeable decrease (or even total loss) in the overall antitumor spectrum and potential. Such dramatic reduction in the bioactivity of these analogs might be ascribed to their steric bulkiness which would probably affect the bioavailability and/or binding with certain targets in the tumor cells. Meanwhile, condensation of the bipyrazole sulfonamide **19** ($R=SO_2NH_2$) with aromatic aldehydes as in **30** ($R=2,4\text{-}F_2$), led to an obvious reduction of the cytotoxic activity. Interestingly, annulation of the diketopyrazoles **8** and **9** into their corresponding pyranopyrazoles **33** and **34** resulted in an obvious improvement in the cytotoxic potential. In particular, when compared to doxorubicin, the analog **34** ($R=SO_2NH_2$) revealed nearly the same activity against the colon HT29, whereas it showed about 55% of the activity against the breast MCF7 cell lines.

DNA-binding activity

DNA-binding assay on TLC-plate In a trial to search for a possible mechanism behind the anticancer efficacy of the most active compounds **16**, **17**, **21**, **26** and **34**, their DNA binding activity was determined. The principle of this method depends on the ability of DNA to migrate after being applied to RP18 F254 TLC plates, predeveloped with ethanol/water mixture (8:2) as an elution system. When DNA was mixed with compounds with which it has been known to intercalate (e.g. ethidium bromide), the complex was retained at the base line using the same elution system, and the spots were visualized after spraying with anisaldehyde reagent (Pezzuto et al. 1991). In the presence of high affinity DNA-intercalator, higher amount of DNA is bound to form a complex that is retained on the base line. On the other hand, inactive compounds caused DNA to leave the origin and move along the plate. The results revealed that, compounds **16**, **17**, **26**, and **34** displayed the highest affinity toward DNA as demonstrated from retention of the DNA-compound's complex at the base line. Meanwhile, the analog **21** showed moderate binding activity (Table 2).

Methyl green-DNA displacement colorimetric assay

This assay (Burres et al. 1992) was used to determine the degree of displacement of methyl green from DNA by the tested compounds colorimetrically, through measuring the decrease in the absorbance of the DNA/methyl green solution. The results are presented in Table 2 as IC_{50}, which is the sample concentration required to produce 50% reduction in the initial absorbance of the DNA-methyl green solution. The results obtained were fairly in agreement with those obtained from the DNA-binding investigation on TLC plates (Table 2). Compounds **16**, **17**, **26**, and **34** displayed a considerable activity ($IC_{50} = 0.059$, 0.051, 0.049 and 0.047 mmol/mL, respectively), as compared with ethidium bromide ($IC_{50} = 0.004$ mmol/mL). Whereas the analog **21** showed moderate activity ($IC_{50} = 0.093$ mmol/mL). Nevertheless, all of them were less active than ethidium bromide.

Here, it should be noted that the DNA-binding activity of the pyrazoles **16**, **17** and the bipyrazolyl derivatives **21**, **26** are going in hand with their structures which are characterized by the presence of the hydroxyl group as a basic component (H-bond donor), beside other H-bond-forming centers, e.g. the arylaminobut-2-en-1-one, sufonamido or thioamide functionalities. Additionally, the sulfonamido group added another credit to analogs **16**, **17**, **21**, and **34**, being a common pharmacophore among diverse anticancer agents including DNA intercalators (Abbate et al. 2004; Al-Said et al. 2010). On the other hand, the DNA-binding capability of the pyranopyrazole analog **34** would be attributed to its binuclear planer structure, together with the encountered H-bond-forming centers. Such favorable structure features would possibly play an important role in enabling the insertion of these molecules between the base pairs or minor/major groove of DNA.

In silico computation of molecular properties (ADME prediction) of the active compounds

Drug-likeness is a term that describes an integrated equilibrium between multiple molecular properties and structure features that define whether a particular compound is comparable to already known drugs. These properties comprise hydrophobicity, electronic distribution, hydrogen-bonding capability, molecule size, and flexibility that would affect the behavior of a molecule in a living system including bioavailability, transport properties, affinity to proteins, reactivity, toxicity, and metabolic stability.

Among the principles applied to evaluate the drug-like properties of a compound, stemmed the Lipinski's rule of five (RO5) (Lipinski et al. 2001) which constitutes a set of key physicochemical properties of drug-like compounds. A molecule likely to be developed as an orally active drug should not violate more than one of the following four criteria: partition coefficient (Clog *P*) ≤ 5, molecular weight (MW) ≤ 500, number of hydrogen bond acceptors ≤10 (HBA), and number of hydrogen bond donors ≤5 (HBD). Violation of more than one of these rules would result in problems in bioavailability upon oral administration. An additional value was developed by Veber et al. (2002) that the number of rotatable bonds (nROTB) should be ≤10 bonds. ROTB is a simple topological measure of a molecule's flexibility and is a good descriptor of oral bioavailability of drugs. In this context, a computational study for the biologically active compounds (**7–9**, **12**, **16**, **17**, **19**, **21**, **22**, **26**, **28**, **30**, **32**, **33**, and **34**) utilizing the Molinspiration online property calculation toolkit (www.molinspiration.com), was carried out to determine the Lipinski's molecular properties and the number of rotatable bonds (nROTB), together with the topographical polar surface area (TPSA; a sum of polar atoms' surfaces). TPSA has been found to be a good descriptor for drug absorption including intestinal absorption, penetrability and bioavailability. Molecules with TPSA values around 140 $Å^2$ or more are expected to exhibit poor intestinal absorption (Veber et al. 2002). Additionally, the percentage of absorption (ABS%)

Table 3 Calculated molecular properties and Lipinski's parameters of the active compounds

Cpd.	Lipinski's parameters[a]					nROTB[g]	TPSA[h]	% ABS[i]	Volume[j]
	CLog *P*[b]	MW[c]	HBA[d]	HBD[e]	Violations[f]				
Rule	≥5	≥500	≥10	≥5	≥1	–	–	–	–
7	1.05	258.28	5	1	0	4	72.20	84.09	232.57
8	1.01	303.27	8	1	0	5	118.02	68.28	255.91
9	−0.25	337.36	8	3	0	5	132.36	63.34	275.29
12	4.53	412.29	5	2	0	5	67.15	85.83	325.90
16	3.23	491.37	8	4	0	6	127.32	65.07	368.62
17	2.58	430.46	8	4	0	6	127.32	65.07	355.67
19	0.69	333.37	8	4	0	3	126.90	65.22	272.76
21	2.03	409.47	8	3	0	4	116.05	68.96	344.55
22	0.72	488.55	11	5	1	5	176.21	48.21	387.27
26	3.95	389.48	6	2	0	5	67.91	85.57	342.09
28	3.65	544.66	9	3	1	8	114.08	69.64	457.34
30	2.99	457.46	8	2	0	5	113.24	69.93	366.00
32	4.89	576.48	8	1	1	6	102.39	73.68	445.82
33	2.04	285.26	7	0	0	2	93.86	76.62	237.66
34	0.78	319.34	7	2	0	2	108.20	71.67	257.05

[a] Molinspiration chemoinformatics property calculator (2014)

[b] Partition coefficient

[c] Molecular weight

[d] Number of H-Bond acceptors (O and N atoms)

[e] Number of H-Bond donors (OH and NH groups)

[f] Number of Rule of 5 violations

[g] Number of rotatable bonds

[h] Topological polar surface area

[i] Absorption %

[j] Molecular volume

calculated as (ABS% = 109–0.345 × TPSA) (Zhao et al. 2002) and the molecular volume (a determinant of the transport characteristics) were determined.

The results presented in Table 3 revealed that all the tested compounds comply with Lipinski's rule of 5, where Clog*P* values ranged between −0.25 to 4.89 (<5), MW range 258–491.37 (<500), HBA range 5–9 (≤10) and HBD range 1–4 (<5). The exception was noted for compound **22** (HBD > 10) and compounds **28** and **32** (MW > 500), which showed only one violation to the RO5, suggesting good drug-likeness properties upon oral administration. Moreover, all the tested compounds showed nROTB values of 2–8 (<10) indicating acceptable molecular flexibility with consequent expected good permeability and oral bioavailability. Additionally, all the evaluated compounds showed TPSA range 67.15–132.36 Å² (<140 Å²), indicating good intestinal absorption, permeability and transport in the cellular plasma membrane; Furthermore, all the tested compounds exhibited a good % ABS ranging from 63.34 to 85.83%, except for the analog **22** (TPSA = 176.21 Å², % ABS = 48.21%). Therefore, these compounds may have a good potential for development as oral agents.

Conclusion

The main objective of this research work was to synthesize some polysubstituted pyrazoles, bipyrazoles and pyranopyrazoles, to be biologically evaluated for their cytotoxic and DNA-binding potentials. In general, better biological activity was confined to compounds comprising the sulfonamido moiety at the N_1 phenyl ring of the pyrazole scaffold. The obtained data revealed that compounds **16**, **17**, **21**, **26**, and **34** showed a considerable broad spectrum cytotoxic potential against the three tested human tumor cell lines, with special effectiveness against the colon HT29 and breast MCF7 cancer cell lines. In particular, compounds **16**, **17**, and **26** displayed double the activity of doxorubicin against colon carcinoma HT29 cell line. DNA-binding studies were in agreement with the obtained antitumor activity, where compounds **16**, **17**, **26**, and **34** displayed the highest affinity. The substitution pattern of these active compounds with multiple H-bond- forming centers would possibly assist the insertion of these molecules between the base pairs of DNA, and hence their cytotoxic potential. In addition, in silico ADME study showed that most of the active compounds comply with Lipinski's RO5 and Veber's criteria, suggesting good drug-likeness properties upon oral administration. Finally, the broad spectrum antitumor potential expressed by the active compounds **16**, **21**, **17**, **26**, and **34**, increases the likelihood of their future derivatization in order to find more active and selective lead structures with potential cytotoxic activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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