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Original article

Synthesis, structure–activity relationships, and in vitro antibacterial and antifungal activity evaluations of novel pyrazole carboxylic and dicarboxylic acid derivatives

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ABSTRACT

A series of pyrazole-3-carboxylic acid and pyrazole-3,4-dicarboxylic acid derivatives were synthesized, the structures were confirmed by their NMR (^{1}H and ^{13}C) and FT-IR spectra, and elemental analyses. The antibacterial and antifungal activities of the compounds against five bacterial and five fungal pathogens were screened using modified agar well diffusion assay. Most of the molecules have inhibitory effects on both standard and clinical *Candida albicans* strains. However, only the molecules **8**, **10**, **21**, and **22** demonstrate some inhibitory effects on *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata* strains. The structure–antifungal activity relationships of the compounds on the *C. albicans* strains were investigated by electron-conformational method. The pharmacophores and antipharmacophores responsible for the inhibition and non-inhibition of the *C. albicans* strains were obtained by electronic and geometrical characteristics of the reactive fragments of the molecules. These fragments along with the associated parameters can be used in designing the future more potent antifungal agents. It has been shown that both the positions of electronegative atoms like F and O in the pyrazole substituents and the amount of the associated charges on such atoms are crucial in regulating the strength of antifungal activity for the *C. albicans* strain.

1. Introduction

The synthesis of pyrazole derivatives that contain a five-membered heterocyclic organic compound with two adjacent nitrogen atoms has great interest in agrochemical, pharmaceutical, and chemical industries [1]. For example, they possess a wide range of bioactivities [1, 2], including antiviral [3], anti-inflammatory [4], anticonvulsant [5], anticancer [6], insecticidal [7], and antifungal [8, 9] activities. In recent years, several drugs including patented ones are developed from the pyrazole derivatives of five-membered ring. For instance, **celecoxib** demonstrates anti-inflammation effect and inhibits COX-2; **rimonabant** functions as cannabinoid receptor and is utilized in obesity treatment; **fomepizole** inhibits alcohol dehydrogenase; and **sildenafil** inhibits phosphodiesterase (Fig. 1).

Modification of the structural profile by altering the 1-, 3-, or 4-position substituent in pyrazole ring affects some bioactivities remarkably [2,10]. The incorporation of trifluoromethyl groups into organic molecules, including pyrazole derivatives, has a potential to modify the bioactivities [11–15].

In continuation of our research efforts of the discovery of novel pyrazole derivatives [16–18], herein we describe synthesis, antibacterial, and antifungal activities of a series of novel pyrazole carboxylic acid and dicarboxylic acid derivatives. The structure–activity relationships of the *Candida albicans* strains have also been studied in terms of electronic and geometrical characteristics by using electron-conformational method. This comprehensive approach of combining the experimental and quantum chemical studies give a chance to predict and to design further novel derivatives.

Nitro group is known to lower solubility of compounds. Therefore, nitro compounds are rarely considered in bioactivity measurements. Since the polar groups present in the novel pyrazole derivatives counteract the insolubility effect of the nitro group, the

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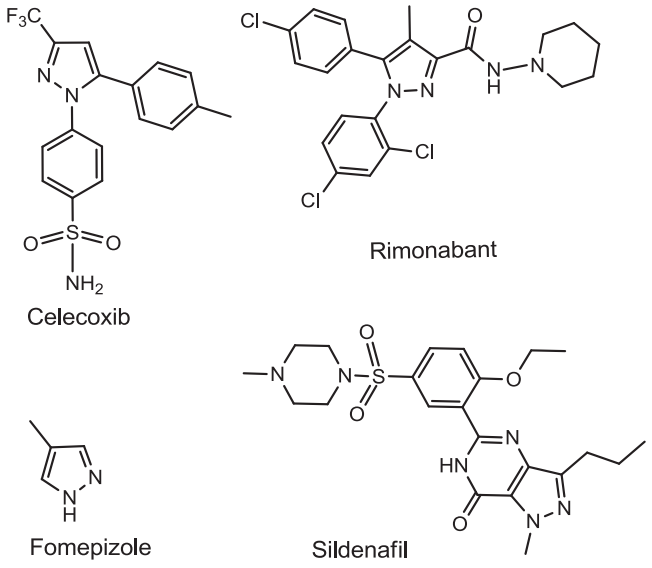


Fig. 1. Several drug molecules which include pyrazole scaffold.

present pyrazole derivatives with a nitro group become soluble in DMSO at 25 °C, allowing antifungal and antibacterial activity measurements of the present molecules.

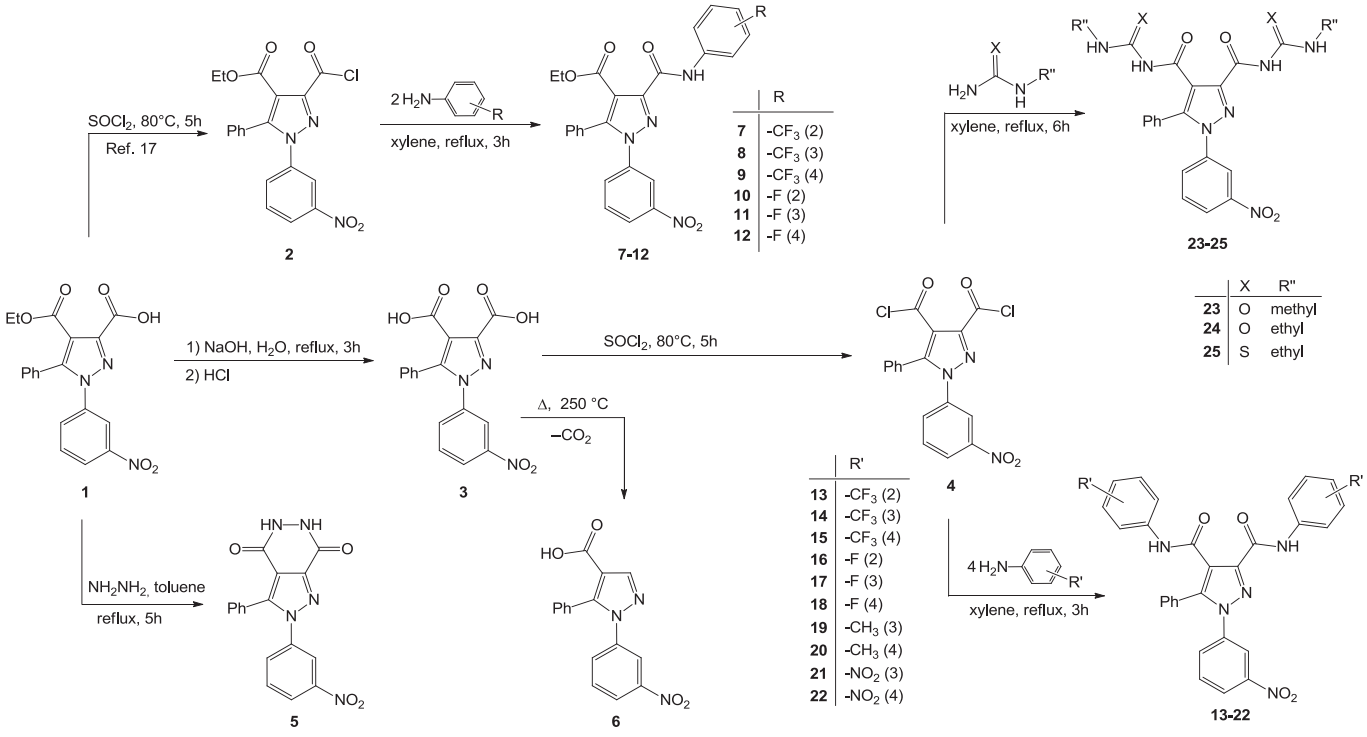
^{1}H and ^{13}C NMR spectroscopy were employed for clarifying chemical structures of the derivatives while FT-IR was applied as a complementary technique for determining their structure via monitoring the frequencies of the characteristic functional groups such as (C=O). The molecular weight of the novel molecules were confirmed by elemental analysis. The bioactivity evaluations were done by both standard strains and species obtained from patients.

2. Results and discussion

2.1. Chemistry

In the current report, the starting compound (**1**) was synthesized via the reaction of furandiones with hydrazones by heating in solventless media [19], and its acid chloride (**2**) was obtained from the reaction with $SOCl_2$ [16]. Then a novel pyrazole-3,4-dicarboxylic acid (**3**) was prepared from the basic hydrolysis of **1** at a high yield (88%). IR spectrum of **3** showed a broad absorption band from 2500 to 3500 cm^{-1} due to OH stretching of −COOH. The absorption bands associated with other functional groups appeared in the expected regions and the absorption values were consistent with our previous reports and literature [16,19]. Pyrazole 3,4-dicarboxylic acid (**3**) was easily converted to its acid chloride (**4**) reacted with excess $SOCl_2$ in solventless media again. The method is easy to perform and gives the product in high yield (82%). Reaction of **1** with anhydrous hydrazine led to the formation of a pyrazolo-pyridazine derivative (**5**) in about 63% yield (See Scheme 1). In this reaction, -NH_2 groups of hydrazine attacks to carbonyl carbons of **1** and in the second stage cyclization occurs with the removal of 1 mol water and 1 mol ethanol. Characteristic NH stretching bands are observed at 3361 cm^{-1} and 3380 cm^{-1} for compound **5**. Considering the reactions and the final products in the current study, the important IR peaks are CH (aromatic), C=O (amide), NH_2, C=C, C=N. The stretching of aromatic CH groups shows frequencies around 3060 cm^{-1} [20]. Broad bands of the NH stretching indicate downward wave-numbers [21]. The IR signals of C=C and C=N appear as a region rather than single sharp peaks. This is explained by several motions such as in plane vibration of C=N [22].

The ^{1}H NMR results depict the successful synthesis of the molecules. In the ^{1}H NMR spectrum of the compound **5** the peaks belonging to NH groups are observed as broad singlet at 12.57 ppm. The compound **6** was obtained from the decarboxylation of **3** at



Scheme 1. The synthetic route of pyrazole carboxylic acid derivatives.

elevated temperature. In this reaction, decarboxylation occurs at the 3 position of the pyrazole ring as previously mentioned in our studies [23]. Compound **6** also shows a singlet peak in the ^{1}H NMR spectrum at 8.23 ppm attributed to CH proton at the 3 position of the pyrazole ring. Then novel pyrazole-3-carboxamides **7**–**12** and pyrazole-3,4-dicarboxamides **13**–**22** were synthesized from the reaction of acid chloride **2** with the corresponding aniline derivatives. The signals of the aromatic protons appear between 8.25 ppm and 6.80 ppm in the ^{1}H NMR spectra. The aromatic protons show various multiplicities such as doublets due to several couplings. The peaks belonging to NH groups are observed in the region between 12.50 ppm and 10.00 ppm. The down-field shift of NH signal is attributed to deshielding because of electron withdrawing ability of the adjacent benzene ring. The ^{1}H resonances of aliphatic groups such as CH_2 and CH_3 are observed in the region between 0.65 ppm and 4.15 ppm. The couplings between these groups led to multiplicities of the resonances.

In the ^{13}C NMR spectrum of compound **6** only one C═O peak was observed at 163.52 ppm. Finally, ureide derivatives **23**–**25** were prepared from the acid dichloride **4** with urea and thiourea derivatives. The ^{13}C NMR and ^{1}H NMR spectra of ureides **(23**–**25)** are also in full agreement with the proposed structures (See Experimental section). In ^{13}C NMR spectra characteristic C═O peaks related to ureas were observed at ~153 ppm and C═S peaks were observed at ~178 ppm. The ^{13}C NMR spectra indicate the signals of aromatic carbons are between 140.00 ppm and 120.00 ppm. The peaks of C═O groups appear closer to 160.00 ppm. All the carbons bonded to N give signals shifted downfield with respect to aromatic carbons, consistent with literature [24]. The ^{13}C NMR signals of $-CF_3$ groups appear around ~125.00 ppm while the fluorinated carbon of the benzene ring gives a downfield signal higher than 150.00 ppm. The ^{13}C NMR signals of aliphatic groups appear between 45.00 ppm and 12.00 ppm. ^{13}C NMR peaks of aliphatics next to benzene ring are relatively shifted with respect to their analogues without benzene in the neighborhood. Such shifts due to benzene rings in the ^{13}C NMR spectra are also consistent with similar results in the case of ^{1}H NMR spectra.

2.2. Antibacterial and antifungal activities

In the present contribution, 21 different pyrazole derivatives were synthesized, the antibacterial and antifungal effects on various bacteria (both gram positive and negative) and fungi were analyzed (Table 1). None of the 21 derivatives depicted antimicrobial effect on gram negative bacteria (*P. aeruginosa*, *Escherichia coli*). This is attributed to intrinsic resistance of the gram negative bacteria. The membrane of the gram negative bacteria functions as a barrier and prevents penetration of the derivative molecules [25]. In order to figure out the antimicrobial effect of the 21 compounds, *Staphylococcus aureus*, *MRSA*, and *E. faecalis* strains were utilized. Some of the derivatives exhibited effect on *S. aureus* and *MRSA*. The 21 molecules did not show any effect on the other gram positive bacteria, *E. faecalis*.

The species *S. aureus* cause several infections including endocarditis, osteomyelitis, and arthritis. *S. aureus* is one of the major reasons of hospital originated nosocomial infections. Methicillin-resistant *S. aureus* (MRSA) shows resistance towards both methicillin and oxacilline. Hospital originated MRSAs having potential to form epidemics are serious threats to public health. Methicillin resistance found in *Staphylococcus*, an intrinsic resistance, cover not only all β-lactam antibiotics, but also depict resistance against most of the antimicrobial agents used in MRSAs [26]. For this reason, there is need for alternative agents.

Compounds **6** and **23** have MIC value of 50 μg/ml on *S. aureus*. The same compounds have MIC values of 25 and 50 μg/ml towards MRSA, respectively. In order to focus on antifungal effects of the molecules of interest, the strains of *Candida glabrata*, *C. albicans*, *Candida tropicalis*, *Candida parapsilosis* were tested. Some of the molecules have remarkable effect on *C. albicans*. *C. albicans* are found as commensal species in various organs of human beings and animals including oral cavity, intestinal tracts, and vaginal cavity.

Table 1
Minimum inhibitory concentration of strains used in agar well diffusion methods.

Compound	Minimum inhibitory concentration (MIC) μg/mL[a]									
	Gram negative bacteria		Gram positive bacteria			Fungi				
	Pa	Ec	Es	Sa	MRSA	Cg	Ca*	Ca**	Ct	Cp
5	–	–	–	–	–	–	–	50	–	–
6	–	–	–	50	25	–	–	25	–	–
7	–	–	–	–	–	–	50	50	–	–
8	–	–	–	–	–	–	50	25	50	–
9	–	–	–	–	–	–	25	25	–	–
10	–	–	–	–	–	50	50	25	–	–
11	–	–	–	–	–	–	50	25	–	–
12	–	–	–	–	–	–	50	50	–	–
13	–	–	–	–	–	–	50	25	–	–
14	–	–	–	–	–	–	–	50	–	–
15	–	–	–	–	–	–	–	50	–	–
16	–	–	–	–	–	–	–	25	–	–
17	–	–	–	–	–	–	–	50	–	–
18	–	–	–	–	–	–	–	25	–	–
19	–	–	–	–	–	–	–	–	–	–
20	–	–	–	–	–	–	–	25	–	–
21	–	–	–	–	–	–	25	25	–	50
22	–	–	–	–	–	–	25	25	50	25
23	–	–	–	50	50	–	25	25	–	–
24	–	–	–	–	–	–	25	25	–	–
25	–	–	–	–	–	–	25	25	–	–

[a] Pa: *P. aeruginosa*, Ec: *E. coli* (Agricultural Research Center Culture Collection), Es: *Enterococcus* spp. (Vancomycin resistance enterococ), Sa: *S. aureus*, MRSA: Methicillin resistance *S. aureus*, Cg: *C. glabrata*, Ca*: *C. albicans* ESOGÜ (Eskisehir Osmangazi University), Ca**: *C. albicans* (Agricultural Research Center Culture Collection), Ct: *C. tropicalis*, Cp: *C. parapsilosis*.

Moreover, *C. albicans* may cause diseases in human beings with weak immune system, having cytotoxic therapy, using antibiotics with broad spectrum [27].

The random usage of antifungals for medical treatment and prolific purposes caused various resistance developments in some fungi species. Some *C. albicans* species may also show resistance towards antifungal drugs. Candida resistance arise from either mutations in the genes responsible for the synthesis of ERGH enzymes affected by the drugs or the extreme expression of CDR1, CDR2, MDR1 type efflux pumping genes. Due to those difficulties and problems in *C. albicans* infection treatments, there is need for alternative medical treatments [28].

In the current study, all the molecules (excluding **19**) showed inhibition effect on the standard *C. albicans* strain (See Table 1). The *C. albicans'* values towards the molecules of interest are within the range of 25–50 μg/ml. The molecules **5**, **6**, **14**–**20** did not exhibit inhibition on the clinical isolate of *C. albicans*. The susceptibility differences between the strains may be based on the origin of yeast. The clinical isolates were obtained from a hospitalized patient at the Eskişehir Osmangazi University hospital, while standard strain was taken from culture collection. The molecules **7**, **8**, **10**–**13** have MIC values of 50 μg/ml, while the other molecules **9**, **21**–**25** have MIC values of 25 μg/ml. *C. parapsilosis*, following *C. albicans*, is the second most isolated yeast from blood cultures in the hospitals [29]. *C. parapsilosis* causes the invasive candida infection. The infection caused by *C. parapsilosis* is related to prostatic tool and catheter in addition to nosocomial dispersion [29]. The MIC values of *C. parapsilosis* in the present study two compounds **21** and **22** are 50 and 25 μg/ml, respectively. In the case of *C. tropicalis* strain, only molecules **8** and **22** showed antimicrobial effects with MIC value of 50 μg/ml. In inhibiting *C. glabrata*, only molecule **10** had the effect with MIC value of 50 μg/ml. The current results are compared to the results obtained by the antibacterial and antifungal agents. Cefotaxime (CTX; 30 μg/disc) for bacteria and nystatin (100 U/disc) for yeast were used as positive control agents. Cefotaxime was not tested against *P. aeroginosa* and *Enterococcus sp*. Cefotaxime did not show activity against MRSA while it exhibited antimicrobial activity against *S. aureus* (35 mm) and *E. coli* (45 mm). Nystatin depicted inhibition against *C. albicans*, *C. tropicalis*, *C. parapsilosis* on 19 mm diameter while against *C. glabrata* on 24 mm.

The molecules of the current report have the highest inhibition effect on the standard and clinical isolates of *C. albicans*. The compounds reported herein are promising candidates for further investigations in finding inhibitors of various bacteria and fungi species.

2.3. Structure–antifungal activity relations

The present molecules demonstrate antifungal activities at different strengths (Table 1) on both the clinical isolate of *C. albicans* (Ca*) and the standard *C. albicans* (Ca**) strain. In this study, the structure–activity relationships (SARs) are investigated by using electron-conformational method (ECM) known also as electron-topological method (ETM) [30–38]. ECM deals with the fine details of the molecules, i.e., geometrical as well as electronic parameters of each atom and bond in a molecule, rather than global characteristics, and thus extracts more information compared with the majority of the other SAR methods. Classical SAR methods can investigate the compounds that are structurally alike. However, ECM can handle diverse molecular structures simultaneously since each atom and each bond in the molecules are represented by numbers rather than atomic types. The main steps of ECM are as follows.

(a) The molecular structure of each compound is determined by geometry optimizations and conformational analyses. For this purpose, we utilized the semi-empirical PM5 quantum chemistry method [39]. All the energetically accessible conformers of each compound are included in the ECM study as separate compounds since the molecules may not be found in their lowest energy conformer while interacting with the bio-receptor.

(b) Each compound is expressed as a square matrix called electron-conformational matrices of contiguity (ECMC). ECMC is symmetric with respect to the diagonal elements. Hence, we only demonstrate the upper part of ECMC in this contribution. The diagonal elements of ECMC are chosen from electronic atomic characteristics of each atom in a molecule. In this current report, the diagonal elements are the atomic charges (Q_i). The non-diagonal elements of ECMC are of two kinds, one of which is for chemical bonds and the other one is for chemically non-bonded atoms. Bond orders (B_{ij}) and interatomic distances (R_{ij}) are utilized here for the chemically bonded and non-bonded atoms, respectively.

(c) ECMC of one of the active compounds is chosen as a template. Each submatrix of the selected ECMC is compared with the each submatrix of the rest of ECMCs within some flexibility limits to reveal the submatrix present in the ECMCs of all active compounds but absent in the ECMCs of all the inactive ones. The found submatrix is called as electron-conformational submatrix of contiguity (ECSC) and corresponds to the activity feature, in other words, to the pharmacophore of the investigated bioactivity. When the ECMC of one of the inactive or weakly active compounds is chosen as a template, inactivity or weak activity features can also be extracted.

In searching ECSC, only three flexibility limits are used initially: one for diagonal elements, one for bond parameters, and one for interatomic distances since each matrix contains huge number of elements $[n\cdot(n+1)/2$, where n is the number of atoms in the molecule], and entering different limits for each parameter is impractical. After revealing the ECSC on a molecule, the flexibility range of its each element is determined finely. The molecule-in parameters in the ECSC are not in the middle of their flexibility ranges in most cases. As we express the parameters and their limits relative to the middle of the flexibility ranges, the features given in this study are template independent. However, we demonstrate the ECSCs on some selected compounds to relate them with the molecular fragments.

The number of molecules demonstrating strong, weak and no inhibition effect on Ca* (Table 1) is six (**9**, and **21**–**25**), six (**7**, **8**, and **10**–**13**), and nine (**5**, **6**, and **14**–**20**), respectively. The feature that is found in the strongly inhibiting molecules and absent in the molecules demonstrating weak and no inhibition effects (SIF*) is considered responsible for demonstrating strong inhibitory effect on Ca* and shown in Fig. 2(a). This feature is formed by four atoms, two of which are chemically bonded. The molecular fragment constituting SIF* is different in **9** from the other strongly inhibiting molecules (**21**–**25**) (Fig. 2(b) and (c)). In the fluorinated molecules like **9**, although it is essential to have a CF_3 moiety at the *para* position for the strong inhibitory effect, this is not alone enough. The other parameters entering SIF* must also associate to the molecules within the given limits in SIF*. For example, CH_3 moiety of the $-(CO)-OCH_2CH_3$ substituent in **9** enters SIF*. When this substituent is replaced by a fluorinated carboxylic acid as in **15**, the molecule does not include SIF* anymore and thus becomes none inhibitory. In **21**–**25**, when the R′ and X substituents (Scheme 1) include N, O or S atom, the parameters associated to the atom G of SIF* are satisfied and thus they inhibit Ca* strongly (Fig. 2(c)). **13**–**20** is weakly or none inhibitory as they contain CH_3 and F as the R′ substituent.

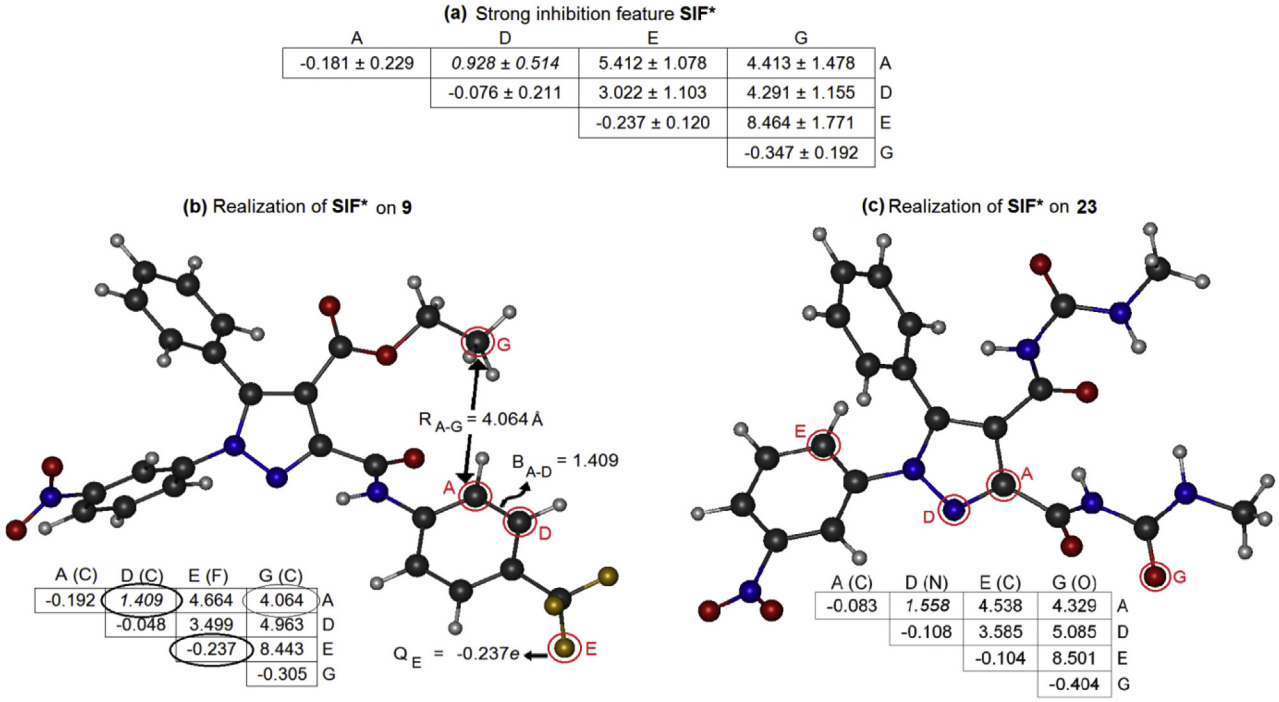


Fig. 2. Strongly inhibiting feature of Ca* (SIF*) and its realization on **9** and **23**. Diagonal elements are atomic charges (Q) whereas non-diagonal elements are either bond orders (B, italic numbers) or interatomic distances (R, plain numbers).

The feature that is found in the weakly inhibiting molecules and absent in the molecules demonstrating strong and no inhibition effects (WIF*) is considered responsible for demonstrating weak inhibitory effect on Ca* and shown in Fig. 3(a). This property is formed by three chemically non-bonded atoms. It is realized when the $-CF_3$ moiety is at the *ortho* (**7** or **13**, Fig. 3(b)) or *meta* (**8**) position rather than the *para* (**9**) position unlike the strongly inhibiting molecules. The feature also enters the molecules in the presence of F atom (rather than $-CF_3$) on the benzene ring irrespective of its position (**10**–**12**).

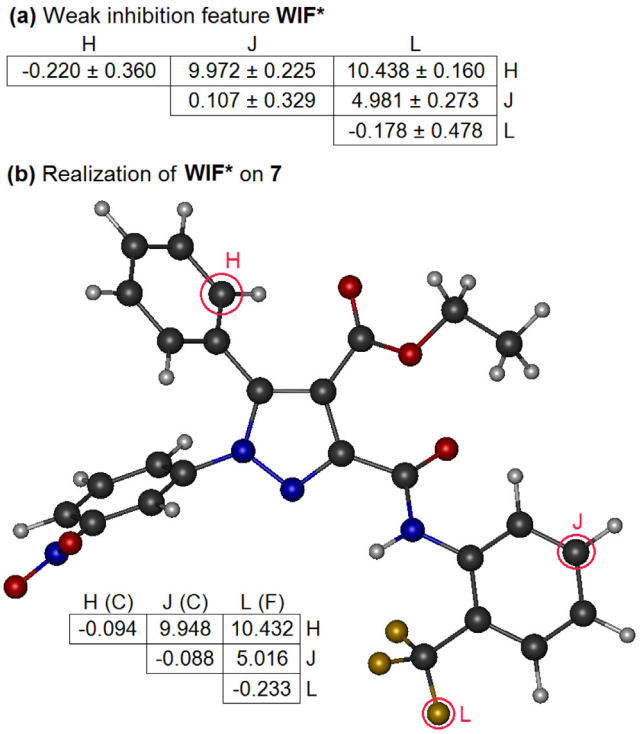


Fig. 3. Weakly inhibiting feature of Ca* (WIF*) and its realization on **7**.

The feature that is found in the none-inhibitory molecules and absent in the molecules demonstrating strong and weak inhibition effects (NIF*) is considered responsible for breaking the inhibitory effect on Ca* and shown in Fig. 4(a). This feature is formed by three chemically non-bonded atoms. Especially, the parameters of NH or OH moiety attached to the >C═O group (see **5** in Fig. 4(b)) are very crucial for the inhibitory activity demonstration. Although all the molecules of interest have such a NH or OH moiety, they become none inhibitory only if the associating parameters are within the values given in Fig. 4(a).

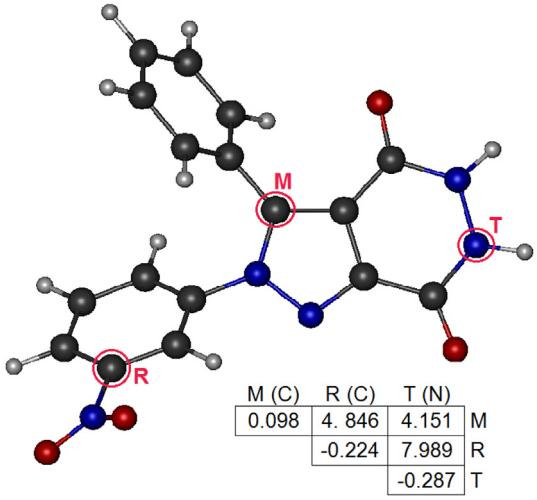
The number of molecules demonstrating strong, weak and no inhibition effect on Ca** (Table 1) is fourteen (**6**, **8**–**11**, **13**, **16**, **18**, and **20**–**25**), six (**5**, **7**, **12**, **14**, **15**, and **17**), and one (**19**), respectively. As there is only one none inhibitory compound on Ca**, the feature responsible for breaking the antifungal activity cannot be determined with the present molecule set. The presence of several related none inhibitory pyrazole derivatives for the Ca** strain is necessary for obtaining more conclusive structure–activity relationships.

The feature that is found in the strongly inhibiting molecules and absent in the molecules demonstrating weak and no inhibition effects (SIF**) is considered responsible for demonstrating strong inhibitory effect on Ca** and shown in Fig. 5(a). This feature is formed by four chemically non-bonded atoms. In all compounds, the atoms entering SIF** (shown in Fig. 5(b) on **9**) are common and their coordinates are very similar to each other. However, their charges are very sensitive to the type of substituent attached to the >C═O moieties. Therefore, the strong inhibition effect on Ca** arise fully from electronic structure of the atoms in the feature and it is difficult to assess if a molecule inhibits Ca** strongly without performing any quantum chemical calculation by just substituent-based analysis.

(a) None inhibition feature **NIF***

M	R	T	
0.092 ± 0.262	4.883 ± 0.117	4.151 ± 0.117	M
	-0.420 ± 0.262	7.989 ± 0.117	R
		-0.548 ± 0.262	T

(b) Realization of **NIF*** on **5**



M (C)	R (C)	T (N)	
0.098	4. 846	4.151	M
	-0.224	7.989	R
		-0.287	T

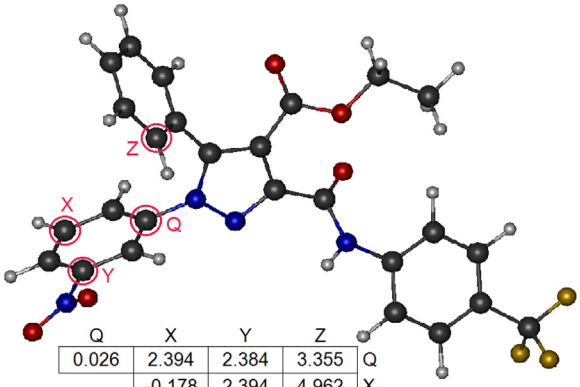
Fig. 4. None inhibiting feature of Ca* (NIF*) that breaks antifungal activity and its realization on **5**.

The feature found in the weakly inhibiting molecules and absent in the molecules demonstrating strong and no inhibition effects (WIF**) is considered responsible for demonstrating weak inhibitory effect on Ca** and shown in Fig. 6(a). This property is established by three chemically non-bonded atoms. As in none inhibitory feature on Ca*, the parameters of NH or OH moiety attached to the >C═O group (see **5** in Fig. 6(b)) appear to be crucial for demonstrating the weak inhibitory activity on Ca**.

(a) Strong inhibition feature **SIF****

Q	X	Y	Z	
-0.037 ± 0.154	2.400 ± 0.235	2.414 ± 0.221	3.458 ± 0.128	Q
	-0.143 ± 0.497	2.418 ± 0.254	4.963 ± 0.157	X
		-0.143 ± 0.494	4.376 ± 0.154	Y
			-0.130 ± 0.575	Z

(b) Realization of **SIF**** on **9**



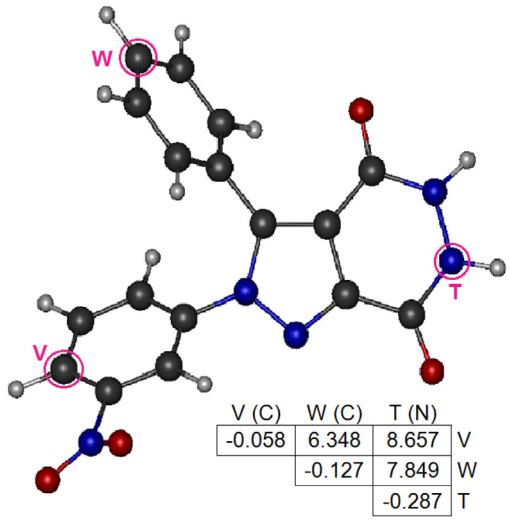
Q	X	Y	Z	
0.026	2.394	2.384	3.355	Q
	-0.178	2.394	4.962	X
		-0.228	4.366	Y
			-0.128	Z

Fig. 5. Strongly inhibiting feature of Ca** (SIF**) and its realization on **9**.

(a) Weak inhibition feature **WIF****

V	W	T	
-0.028 ± 0.087	6.347 ± 0.445	8.605 ± 0.517	V
	-0.143 ± 0.114	7.868 ± 0.442	W
		-0.284 ± 0.081	T

(b) Realization of **WIF**** on **5**



V (C)	W (C)	T (N)	
-0.058	6.348	8.657	V
	-0.127	7.849	W
		-0.287	T

Fig. 6. Weakly inhibiting feature of Ca** (WIF**) and its realization on **5**.

3. Experimental protocols

3.1. Materials and methods

Some aniline derivatives was distilled (*m*-toluidine) or recrystallized (*p*-toluidine, *m*-nitroaniline and *p*-nitroaniline) before use; all other reagents are commercially available and were used without further purification. The solvents used were of analytical grade. Melting points were determined in open glass capillaries using Barnstead Electrothermal 9200 melting point apparatus (Electrothermal Co, Essex, UK) and are uncorrected. Infrared spectra (IR) were recorded on Bruker Optics, Vertex 70 Fourier Transform Infrared Spectrometer (FT-IR) equipped with an ATR (Attenuated Total Reflection) device and the data are reported in reciprocal centimeters (cm^{-1}) (Bruker Optik GmbH, Ettlingen, Germany). ^{1}H NMR and ^{13}C NMR spectra were scanned on Jeol-500 MHz spectrometer (Jeol, Tokyo, Japan) using tetramethylsilane (TMS) as internal standard and using one or two of the following solvents, DMSO-d_6 and $CDCl_3$. Chemical shifts are given in δ, ppm. Splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet. Elemental analyses (C, H, and N) were performed on a Leco CHNS-932 elemental analyser (LECO Corporation, Saint Joseph, Michigan, USA). Follow up of the reactions and checking the purity of the compounds was made by thin layer chromatography (TLC) on silicagel precoated aluminum sheets (Kieselgel 60F 254 of E. Merck, Darmstadt, Germany). Compounds were visualized by Camag TLC devices (Camag, Upland, CA, USA) UV (254 and 366 nm). Compounds **1** and **2** were prepared following previously published reaction conditions [16,19].

3.2. Synthesis

3.2.1. 1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxylic acid (*3*)

A solution of **1** (0.381 g, 1 mmol) and sodium hydroxide (0.1 g, 2.5 mmol) in 30 mL water was refluxed at 100 °C for 3 h. After

solution was cooled down to room temperature it was stirred for a while by adding HCl solution (1.5 mL d. HCl in 20 mL water). The white precipitate was filtered and washed with water again. The residual solid was crystallized in EtOH/H_2O (1:3) mixture to afford title compound **3** as white needles. Yield: 88%; m.p.: 207–208 °C; IR (ν, cm^{-1}): 3224–2474 (OH, COOH), 3069 (CH, aromatic), 1707 (C=O, acid), 1607–1427 (C=C and C=N); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 8.21-7.34 (m, 9H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 164.07 and 163.30 (C=O, acid), 147.94 (C–NO_2), 145.19, (pyrazole C-3), 145.06 (pyrazole C-5), 120.70 (pyrazole C-4), 139.36, 131.98, 130.84, 130.46, 129.94, 128.69, 127.92, 123.54, 116.39. Anal. calcd. for $C_{17}H_{11}N_3O_6$ (353.29 g/mol): C, 57.80; H, 3.14; N, 11.89. Found: C, 57.68; H, 3.17; N, 11.85.

3.2.2. 1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarbonyl dichloride (**4**)

3 (0.353 g, 1 mmol) and excessive amount of thionyl chloride ($SOCl_2$) was heated in 80 °C for 5 h. After the completion of the chlorination, excess $SOCl_2$ were evaporated. Finally, the crude product was recrystallized from a mixture of ether/hexane (5:1). Yield: 82%; m.p.: 58–60 °C; IR (ν, cm^{-1}): 3084 (CH, aromatic), 1754 (C=O, acyl), 1623–1451 (C=C and C=N). ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 8.21–7.33 (m, 9H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 164.04 and 163.26 (C=O, acyl), 147.91 (C–NO_2), 144.94 (pyrazole C-3), 144.84 (pyrazole C-5), 120.66 (pyrazole C-4), 139.27, 131.95, 130.84, 130.38, 129.96, 128.70, 127.73, 123.54, 116.33. Anal. calcd. for $C_{17}H_9Cl_2N_3O_4$ (390.18 g/mol): C, 52.33; H, 2.32; N, 10.77. Found: C, 52.24; H, 2.38; N, 10.81.

3.2.3. 2-(3-nitrophenyl)-3-phenyl-5,6-dihydro-2H-pyrazolo[3,4-d] pyridazine-4,7-dione (**5**)

0.381 g (1 mmol) compound **1** was dissolved in 10 ml dry toluene, and anhydrous hydrazine was added at 1/1 mol rate. The mixture was refluxed at 110 °C about 5 h. Precipitate yellow product was filtered and purified from ethanol/H_2O (2:1) mixture by crystallization. Yield: 63%; m.p.: 307–308 °C; IR (ν, cm^{-1}): 3317 and 3189 (NH), 3056 (CH, aromatic), 1661 (C=O), 1607–1441 (C=C and C=N), 1346 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 12.57 (br, s, 2H, 2NH), 8.30–7.19 (m, 9H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 164.74 and 161.37 (C=O, pyridazine dione), 149.36 (C–NO_2), 147.61 (pyrazole C-3), 145.01 (pyrazole C-5), 121.28 (pyrazole C-4), 139.82, 132.44, 130.71, 130.06, 128.20, 127.92, 127.74, 122.34, 119.98. Anal. calcd. for $C_{17}H_{11}N_5O_4$ (349.30 g/mol): C, 58.45; H, 3.17; N, 18.32. Found: C, 58.38; H, 3.18; N, 18.35.

3.2.4. 1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-4-carboxylic acid (**6**)

0.353 g (1 mmol) pyrazole-3,4 dicarboxylic acid **(3)** was heated at 250 °C until gas exiting finished. Solid at the bottom was washed with ether and water, respectively. The crude product was purified from xylene by crystallization. Yield: 70%; m.p.: 203–204 °C; IR (ν, cm^{-1}): 3100–2550 (OH, COOH), 1675 (C=O, acid), 1608–1444 (C=C and C=N), 1346 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 8.23 (s, 1H, pyrazole CH=N), 8.18–6.94 (m, 9H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 163.52 (C=O, acid), 147.77 (C–NO_2), 145.58 (pyrazole C-3), 143.05 (pyrazole C-5), 120.16 (pyrazole C-4), 139.73, 131.52, 130.59, 129.49, 128.90, 128.24, 126.07, 122.81, 114.95. Anal. calcd. for $C_{16}H_{11}N_3O_4$ (309.28 g/mol): C, 52.14; H, 3.58; N, 13.59. Found: C, 52.05; H, 3.63; N, 13.60.

3.2.5. General procedure for the synthesis of pyrazole-3-carboxamides and pyrazole-3,4-dicarboxamides (**7**–**22**)

1 mmol **2** or **4** was dissolved in approximately 10 mL of dry xylene and for pyrazole-3-carboxamides 2 mmol, and for pyrazole-3,4-dicarboxamides 4 mmol aryl amine was added to this solution. Mixture was refluxed at 140 °C 3 h and the solvent was evaporated under vacuum. The crude product so obtained was washed with water and recrystallized from an appropriate solvent.

3.2.6. Ethyl 1-(3-nitrophenyl)-5-phenyl-3-((2-(trifluoromethyl) phenyl)carbamoyl)-1H-pyrazole-4-carboxylate (**7**)

Synthesized from **2** (0.4 g, 1 mmol) and 2-(trifluoromethyl) aniline (0.262 ml, 2 mmol) according to the general procedure. The crude product was purified by crystallization from methanol. Yield: 89%; m.p.: 161–162 °C; IR (ν, cm^{-1}): 3415 and 3126 (NH), 3100 (CH, aromatic), 2990 (CH, aliphatic), 1696 (C=O, amide), 1592–1451 (C=C and C=N), 1350 (N–O sym.), 1248 and 1067 (C–O–C asym. and sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.43 (s, 1H, NH), 8.27–7.42 (m, 13H, ArH), 4.13 (q, J = 7.1 Hz, 2H, OCH_2), 1.06 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 162.32 (C=O, ester), 160.32 (C=O, amide), 147.76 (C–NO_2), 146.86 (pyrazole C-3), 145.35 (pyrazole C-5), 125.15 (CF_3), 120.62 (pyrazole C-4), 60.66 (OCH_2), 13.61 (CH_3), 139.01, 134.88, 133.22, 131.77, 130.57, 130.18, 129.77, 129.63, 128.46, 127.42, 127.12, 126.52, 124.93, 123.38, 114.37. Anal. calcd. for $C_{26}H_{19}F_3N_4O_5$ (524.45 g/mol): C, 59.54; H, 3.65; N, 10.68. Found: C, 59.48; H, 3.69; N, 10.69.

3.2.7. Ethyl 1-(3-nitrophenyl)-5-phenyl-3-((3-(trifluoromethyl) phenyl)carbamoyl)-1H-pyrazole-4-carboxylate (**8**)

Synthesized from **2** (0.4 g, 1 mmol) and 3-(trifluoromethyl) aniline (0.276 ml, 2 mmol) according to the general procedure. The crude product was purified by crystallization from methanol. Yield: 92%; m.p.: 144–145 °C; IR (ν, cm^{-1}): 3281 and 3216 (NH), 3045 (CH, aromatic), 2992 (CH, aliphatic), 1693 and 1660 (C=O, amide), 1605–1448 (C=C and C=N), 1341 (N–O sym.), 1247 and 1069 (C–O–C asym. and sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.06 (s, 1H, NH), 8.27–7.39 (m, 13H, ArH), 4.10 (q, J = 7.1 Hz, 2H, OCH_2), 1.00 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.71 (C=O, ester), 160.21 (C=O, amide), 147.96 (C–NO_2), 147.75 (pyrazole C-3), 145.47 (pyrazole C-5), 125.43 (CF_3), 120.50 (pyrazole C-4), 60.46 (OCH_2), 13.57 (CH_3), 139.40, 138.92, 131.73, 130.62, 130.22, 130.10, 129.82, 129.65, 129.33, 128.46, 127.31, 123.39, 120.29, 115.84, 113.66. Anal. calcd. for $C_{26}H_{19}F_3N_4O_5$ (524.45 g/mol): C, 59.54; H, 3.65; N, 10.68. Found: C, 59.45; H, 3.68; N, 10.66.

3.2.8. Ethyl 1-(3-nitrophenyl)-5-phenyl-3-((4-(trifluoromethyl) phenyl)carbamoyl)-1H-pyrazole-4-carboxylate (**9**)

Synthesized from **2** (0.4 g, 1 mmol) and 4-(trifluoromethyl)aniline (0.256 ml, 2 mmol) according to the general procedure. The crude product was purified by crystallization from methanol. Yield: 84%; m.p.: 174–175 °C; IR (ν, cm^{-1}): 3267 and 3197 (NH), 3057 (CH, aromatic), 2935 (CH, aliphatic), 1696 and 1667 (C=O, amide), 1606–1448 (C=C and C=N), 1320 (N–O sym.), 1213 and 1062 (C–O–C asym. and sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.09 (s, 1H, NH), 8.27–7.39 (m, 13H, ArH), 4.10 (q, J = 7.1 Hz, 2H, OCH_2), 1.00 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.68 (C=O, ester), 160.29 (C=O, amide), 148.11 (C–NO_2), 147.75 (pyrazole C-3), 145.51 (pyrazole C-5), 123.72 (CF_3), 120.49 (pyrazole C-4), 60.45 (OCH_2), 13.60 (CH_3), 142.22, 138.92, 131.73, 130.63, 130.23, 129.82, 128.46, 127.31, 126.15, 126.11, 123.39, 119.71, 113.58. Anal. calcd. for $C_{26}H_{19}F_3N_4O_5$ (524.45 g/mol): C, 59.54; H, 3.65; N, 10.68. Found: C, 59.42; H, 3.63; N, 10.72.

3.2.9. Ethyl 3-((2-fluorophenyl)carbamoyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-4-carboxylate (**10**)

Synthesized from **2** (0.4 g, 1 mmol) and 2-fluoroaniline (0.214 ml, 2 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 90%; m.p.: 141–142 °C; IR (ν, cm^{-1}): 3295 and 3186 (NH), 3054 (CH,

aromatic), 2979 (CH, aliphatic), 1669 (C=O, amide), 1616–1451 (C=C and C=N), 1347 (N–O sym.), 1214 and 1075 (C–O–C asym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.56 (s, 1H, NH), 8.27–7.25 (m, 13H, ArH), 4.12 (q, J = 7.0 Hz, 2H, OCH_2), 1.03 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 162.10 (C=O, ester), 159.74 (C=O, amide), 155.78 (C–F), 153.32 (C–NO_2), 147.75 (pyrazole C-3), 147.55 (pyrazole C-5), 120.50 (pyrazole C-4), 60.55 (OCH_2), 13.57 (CH_3), 145.42, 139.00, 131.69, 130.59, 130.21, 129.76, 128.45, 127.46, 124.40, 123.33, 115.84, 115.65, 113.79. Anal. calcd. for $C_{25}H_{19}FN_4O_5$ (474.44 g/mol): C, 63.29; H, 4.04; N, 11.81. Found: C, 63.21; H, 4.07; N, 11.80.

3.2.10. Ethyl 3-((3-fluorophenyl) carbamoyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-4-carboxylate (**11**)

Synthesized from **2** (0.4 g, 1 mmol) and 3-fluoroaniline (0.149 ml, 2 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 95%; m.p.: 154–155 °C; IR (ν, cm^{-1}): 3275 and 3214 (NH), 3032 (CH, aromatic), 2992 (CH, aliphatic), 1670 (C=O, amide), 1607–1446 (C=C and C=N), 1331 (N–O sym.), 1221 and 1086 (C–O–C asym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.93 (s, 1H, NH), 8.27–6.95 (m, 13H, ArH), 4.11 (q, J = 7.1 Hz, 2H, OCH_2), 1.01 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 163.26 (C–F), 161.70 (C=O, ester), 160.03 (C=O, amide), 148.19 (C–NO_2), 147.74 (pyrazole C-3), 145.45 (pyrazole C-5), 120.47 (pyrazole C-4), 60.43 (OCH_2), 13.60 (CH_3), 140.41, 140.30, 138.92, 131.72, 130.63, 130.52, 130.42, 130.21, 129.80, 128.46, 127.33, 123.37, 115.54, 113.57. Anal. calcd. for $C_{25}H_{19}FN_4O_5$ (474.44 g/mol): C, 63.29; H, 4.04; N, 11.81. Found: C, 63.18; H, 4.08; N, 11.85.

3.2.11. Ethyl 3-((4-fluorophenyl)carbamoyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-4-carboxylate (**12**)

Synthesized from **2** (0.4 g, 1 mmol) and 4-fluoroaniline (0.198 ml, 2 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 88%; m.p.: 144–145 °C; IR (ν, cm^{-1}): 3276 and 3216 (NH), 3061 (CH, aromatic), 2970 (CH, aliphatic), 1674 (C=O, amide), 1625–1448 (C=C and C=N), 1341 (N–O sym.), 1250 and 1081 (C–O–C asym. and sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.77 (s, 1H, NH), 8.27–7.20 (m, 13H, ArH), 4.10 (q, J = 7.1 Hz, 2H, OCH_2), 1.01 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.79 (C=O, ester), 159.66 (C=O, amide), 157.14 (C–F), 148.40 (C–NO_2), 147.75 (pyrazole C-3), 145.35 (pyrazole C-5), 120.43 (pyrazole C-4), 60.41 (OCH_2), 13.62 (CH_3), 138.96, 135.07, 131.68, 130.61, 130.20, 129.78, 128.46, 127.39, 123.31, 121.63, 121.55, 115.48, 115.26, 113.58. Anal. calcd. for $C_{25}H_{19}FN_4O_5$ (474.44 g/mol): C, 63.29; H, 4.04; N, 11.81. Found: C, 63.19; H, 4.09; N, 11.87.

3.2.12. 1-(3-nitrophenyl)-5-phenyl-N^3,N^4-bis(2-(trifluoromethyl) phenyl)-1H-pyrazole-3,4-dicarboxamide (**13**)

Synthesized from **4** (0.39 g, 1 mmol) and 2-(trifluoromethyl) aniline (0.524 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from methanol. Yield: 65%; m.p.: 164–165 °C; IR (ν, cm^{-1}): 3380 (NH), 3036 (CH, aromatic), 1695 and 1662 (C=O, amide), 1591–1453 (C=C and C=N), 1348 (N–O sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.09 and 10.47 (s, 2H, 2NH), 8.33–7.40 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.31 and 160.94 (C=O, amide), 147.83 (C–NO_2), 146.43 (pyrazole C-3), 143.10 (pyrazole C-5), 126.09 and 126.05 (CF_3), 120.68 (pyrazole C-4), 139.05, 135.13, 134.68, 133.39, 132.87, 131.79, 130.57, 130.13, 129.57, 129.48, 129.45, 128.31, 128.08, 127.39, 126.63, 126.59, 126.48, 123.49, 122.57, 122.36, 118.58. Anal. calcd. for $C_{31}H_{19}F_6N_5O_4$ (639.50 g/mol): C, 58.22; H, 2.99; N, 10.95. Found: C, 58.15; H, 3.04; N, 10.95.

3.2.13. 1-(3-nitrophenyl)-5-phenyl-N^3,N^4-bis(3-(trifluoromethyl) phenyl)-1H-pyrazole-3,4-dicarboxamide (**14**)

Synthesized from **4** (0.39 g, 1 mmol) and 3-(trifluoromethyl) aniline (0.552 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 73%; m.p.: 210–211 °C; IR (ν, cm^{-1}): 3371 (NH), 3073 (CH, aromatic), 1683 and 1660 (C=O, amide), 1601–1447 (C=C and C=N), 1356 (N–O sym.); ^{1}H NMR (500 MHz, DMSO-d_6/$CDCl_3$) δ_H (ppm): 11.88 and 10.56 (s, 2H, 2NH), 8.23–7.23 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6/$CDCl_3$) δ_C (ppm): 161.52 and 159.94 (C=O, amide), 148.14 (C–NO_2), 147.65 (pyrazole C-3), 143.46 (pyrazole C-5), 125.10 and 125.06 (CF_3), 120.37 (pyrazole C-4), 139.45, 139.30, 138.36, 131.15, 130.37, 130.17, 129.78, 129.61, 129.47, 128.64, 128.31, 124.59, 123.19, 123.13, 122.94, 122.90, 121.16, 121.13, 120.12, 120.08, 118.85. Anal. calcd. for $C_{31}H_{19}F_6N_5O_4$ (639.50 g/mol): C, 58.22; H, 2.99; N, 10.95. Found: C, 58.11; H, 3.02; N, 10.97.

3.2.14. 1-(3-nitrophenyl)-5-phenyl-N^3,N^4-bis(4-(trifluoromethyl) phenyl)-1H-pyrazole-3,4-dicarboxamide (**15**)

Synthesized from **4** (0.39 g, 1 mmol) and 4-(trifluoromethyl) aniline (0.512 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 84%; m.p.: 218–219 °C; IR (ν, cm^{-1}): 3342 (NH), 3006 (CH, aromatic), 1671 (C=O, amide), 1607–1447 (C=C and C=N), 1346 (N–O sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.10 and 10.94 (s, 2H, 2NH), 8.31–7.17 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.38 and 160.22 (C=O, amide), 148.25 (C–NO_2), 144.66 (pyrazole C-3), 144.56 (pyrazole C-5), 125.75 and 125.65 (CF_3), 120.58 (pyrazole C-4), 142.86, 142.29, 139.36, 132.09, 131.08, 130.15, 129.24, 129.12, 128.55, 127.70, 126.45, 126.27, 123.88, 123.59, 120.98, 120.85, 119.53. Anal. calcd. for $C_{31}H_{19}F_6N_5O_4$ (639.50 g/mol): C, 58.22; H, 2.99; N, 10.95. Found: C, 58.16; H, 3.04; N, 10.98.

3.2.15. N^3,N^4-bis(2-fluorophenyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxa-mide (**16**)

Synthesized from **4** (0.39 g, 1 mmol) and 2-fluoroaniline (0.428 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 78%; m.p.: 213–215 °C; IR (ν, cm^{-1}): 3371 (NH), 3061 (CH, aromatic), 1660 (C=O, amide), 1614–1452 (C=C and C=N), 1347 (N–O sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.31 and 10.51 (s, 2H, 2NH), 8.31–7.12 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 164.99 and 164.66 (C–F), 160.85 and 160.12 (C=O, amide), 147.94 (C–NO_2), 146.67 (pyrazole C-3), 143.21 (pyrazole C-5), 120.79 (pyrazole C-4), 139.18, 131.95, 130.70, 130.30, 129.62, 128.53, 128.38, 127.04, 126.35, 124.65, 124.63, 124.48, 123.61, 123.54, 118.79, 116.12, 115.96, 115.58, 115.42. Anal. calcd. for $C_{29}H_{19}F_2N_5O_4$ (539.49 g/mol): C, 58.22; H, 2.99; N, 10.95. Found: C, 58.15; H, 3.01; N, 10.97.

3.2.16. N^3,N^4-bis(3-fluorophenyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxa-mide (**17**)

Synthesized from **4** (0.39 g, 1 mmol) and 3-fluoroaniline (0.298 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 86%; m.p.: 228–229 °C; IR (ν, cm^{-1}): 3355 and 3199 (NH), 3058 (CH, aromatic), 1682 and 1656 (C=O, amide), 1611–1445 (C=C and C=N), 1349 (N–O sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.98 and 10.77 (s, 2H, 2NH), 8.29–6.89 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.17 and 161.05 (C–F), 160.78 and 159.74 (C=O, amide), 147.89 (C–NO_2), 144.29 (pyrazole C-3), 144.24 (pyrazole C-5), 120.49 (pyrazole C-4), 139.04, 131.75, 130.72, 130.48, 130.41, 130.36, 130.29, 129.83, 129.74, 128.90, 128.75, 128.21, 127.46, 123.49, 120.21, 116.47, 116.46, 115.06, 115.04. Anal. calcd. for

$C_{29}H_{19}F_2N_5O_4$ (539.49 g/mol): C, 58.22; H, 2.99; N, 10.95. Found: C, 58.12; H, 3.03; N, 10.93.

3.2.17. N^3,N^4-bis(4-fluorophenyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxa-mide (**18**)

Synthesized from **4** (0.39 g, 1 mmol) and 4-fluoroaniline (0.396 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 74%; m.p.: 232–233 °C; IR (ν, cm^{-1}): 3362 and 3216 (NH), 3082 (CH, aromatic), 1688 and 1660 (C=O, amide), 1633–1451 (C=C and C=N), 1344 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6/$CDCl_3$) δ_H (ppm): 11.71 and 10.37 (s, 2H, 2NH), 8.17–6.90 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6/$CDCl_3$) δ_C (ppm): 161.21 and 159.59 (C=O, amide), 158.40 and 157.72 (C–F), 148.05 (C–NO_2), 147.41 (pyrazole C-3), 143.64 (pyrazole C-5), 120.32 (pyrazole C-4), 139.32, 135.03, 133.78, 131.27, 130.32, 130.23, 129.65, 128.94, 128.54, 128.19, 123.32, 123.26, 123.14, 121.63, 121.56, 118.83, 115.50, 115.32, 115.16. Anal. calcd. for $C_{29}H_{19}F_2N_5O_4$ (539.49 g/mol): C, 58.22; H, 2.99; N, 10.95. Found: C, 58.13; H, 3.02; N, 10.98.

3.2.18. 1-(3-nitrophenyl)-5-phenyl-N^3,N^4-di-m-tolyl-1H-pyrazole-3,4-dicarboxamide (**19**)

Synthesized from **4** (0.39 g, 1 mmol) and freshly distilled *m*-toluidine (0.437 ml, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 95%; m.p.: 183–184 °C; IR (ν, cm^{-1}): 3351 and 3248 (NH), 3061 (CH, aromatic), 2971 (CH, aliphatic), 1659 (C=O, amide), 1618–1456 (C=C and C=N), 1345 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.84 and 10.48 (s, 2H, 2NH), 8.28–6.87 (m, 17H, ArH), 2.31 and 2.26 (s, 6H, $2CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 160.29 and 159.82 (C=O, amide), 147.91 (C–NO_2), 144.43 (pyrazole C-3), 144.39 (pyrazole C-5), 120.34 (pyrazole C-4), 21.21 (CH_3), 139.18, 139.02, 138.16, 137.99, 137.95, 131.60, 130.67, 129.95, 129.62, 128.68, 128.60, 128.56, 127.89, 124.91, 124.21, 123.31, 121.24, 120.30, 119.74, 117.95, 116.49. Anal. calcd. for $C_{31}H_{25}N_5O_4$ (531.56 g/mol): C, 70.04; H, 4.74; N, 13.18. Found: C, 69.95; H, 4.77; N, 13.22.

3.2.19. 1-(3-nitrophenyl)-5-phenyl-N^3,N^4-di-p-tolyl-1H-pyrazole-3,4-dicarboxamide (**20**)

Synthesized from **4** (0.39 g, 1 mmol) and *p*-toluidine (0.451 g, 4 mmol) according to the general procedure. The crude product was purified by crystallization from toluene. Yield: 88%; m.p.: 197–198 °C; IR (ν, cm^{-1}): 3347 (NH), 3029 (CH, aromatic), 2973 (CH, aliphatic), 1669 (C=O, amide), 1608–1451 (C=C and C=N), 1345 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 10.89 and 10.52 (s, 2H, 2NH), 8.27–7.09 (m, 17H, ArH), 2.28 and 2.24 (s, 6H, $2CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 160.02 and 159.83 (C=O, amide), 147.87 (C–NO_2), 144.59 (pyrazole C-3), 144.41 (pyrazole C-5), 120.36 (pyrazole C-4), 20.53 and 20.47 (CH_3), 139.17, 136.55, 135.65, 133.28, 132.46, 131.62, 130.63, 129.97, 129.55, 129.09, 128.59, 127.95, 123.29, 120.81, 120.10, 119.29. Anal. calcd. for $C_{31}H_{25}N_5O_4$ (531.56 g/mol): C, 70.04; H, 4.74; N, 13.18. Found: C, 70.01; H, 4.78; N, 13.21.

3.2.20. N^3,N^4,1-tris(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxamide (**21**)

Synthesized from **4** (0.39 g, 1 mmol) and 3-nitroaniline (0.558 g, 4 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol/DMF (3:1). Yield: 81%; m.p.: 291–292 °C; IR (ν, cm^{-1}): 3314 (NH), 3006 (CH, aromatic), 1665 (C=O, amide), 1609–1451 (C=C and C=N), 1342 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.20 and 11.09 (s, 2H, 2NH), 8.82–7.42 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.19 and 159.80 (C=O, amide), 147.96, 147.87 and 144.26 (C–NO_2), 144.07 (pyrazole C-3), 140.04 (pyrazole C-5), 120.49 (pyrazole C-4), 139.51, 138.97, 131.73, 130.74, 130.28, 130.11, 129.79, 128.81, 128.42, 127.21, 126.61, 125.19, 123.55, 120.10, 118.55, 118.15, 114.77, 113.20. Anal. calcd. for $C_{29}H_{19}N_7O_8$ (593.50 g/mol): C, 58.69; H, 3.23; N, 16.52. Found: C, 58.57; H, 3.19; N, 16.52.

3.2.21. 1-(3-nitrophenyl)-N^3,N^4-bis(4-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxa-mide (**22**)

Synthesized from **4** (0.39 g, 1 mmol) and 4-nitroaniline (0.564 g, 4 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol/DMF (3:1). Yield: 89%; m.p.: 320–321 °C; IR (ν, cm^{-1}): 3339 and 3214 (NH), 3049 (CH, aromatic), 1671 (C=O, amide), 1634–1450 (C=C and C=N), 1324 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.29 and 11.17 (s, 2H, 2NH), 8.32–7.40 (m, 17H, ArH); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 161.27 and 159.89 (C=O, amide), 147.91, 145.02 and 144.09 (C–NO_2), 144.58 (pyrazole C-3), 144.48 (pyrazole C-5), 120.59 (pyrazole C-4), 142.81, 142.44, 138.92, 131.80, 130.77, 129.89, 129.80, 128.83, 127.12, 125.06, 124.75, 123.65, 120.40, 120.12, 119.03. Anal. calcd. for $C_{29}H_{19}N_7O_8$ (593.50 g/mol): C, 58.69; H, 3.23; N, 16.52. Found: C, 58.55; H, 3.27; N, 16.56.

3.2.22. General procedure for the synthesis of diureide derivatives (**23**–**25**)

A mixture of the acid dichloride **4** (0.39 g, 1 mmol) and *N*-alkyl ureas or thioureas (2 mmol) was refluxed at 140 °C in xylene for 6 h. After solvent had been evaporated, the formed crude product was recrystallized from methanol or ethanol.

3.2.23. N^3,N^4-bis(methylcarbamoyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxa-mide (**23**)

Synthesized from **4** (0.39 g, 1 mmol) and *N*-methylurea (0.153 g, 2 mmol) according to the general procedure. The crude product was purified by crystallization from methanol. Yield: 72%; m.p.: 220–221 °C; IR (ν, cm^{-1}): 3319 (NH), 3054 (CH, aromatic), 2970 (CH, aliphatic), 1708 and 1671 (C=O, ureide), 1637–1451 (C=C and C=N), 1348 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 12.37 and 11.39 (s, 2H, 2NH), 10.88 and 9.02 (s, 2H, 2NH), 8.34–7.36 (m, 9H, ArH), 2.86 and 2.66 (d, $J = 6.8$ Hz, 6H, $2CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 162.53 and 162.15 (C=O, amide), 153.77 and 153.36 (C=O, ureide), 147.96 (C–NO_2), 147.68 (pyrazole C-3), 143.26 (pyrazole C-5), 120.75 (pyrazole C-4), 26.21 (NCH_3), 138.92, 131.93, 130.95, 130.54, 129.70, 128.87, 128.32, 123.54, 116.07. Anal. calcd. for $C_{21}H_{19}N_7O_6$ (465.42 g/mol): C, 54.19; H, 4.11; N, 21.07. Found: C, 54.10; H, 4.15; N, 21.09.

3.2.24. N^3,N^4-bis(ethylcarbamoyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarboxa-mide (**24**)

Synthesized from **4** (0.39 g, 1 mmol) and *N*-ethylurea (0.179 g, 2 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 65%; m.p.: 227–228 °C; IR (ν, cm^{-1}): 3355 and 3226 (NH), 3062 (CH, aromatic), 2971 (CH, aliphatic), 1702 and 1659 (C=O, ureide), 1603–1450 (C=C and C=N), 1347 (N–O sym.); ^1H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 12.40 and 11.55 (s, 2H, 2NH), 10.42 (br, s, 2H, 2NH), 8.33–7.35 (m, 9H, ArH), 3.26 and 3.16 (pentet, $J = 6.8$ Hz, 4H, 2NH$\underline{CH_2}CH_3$), 1.12 and 1.05 (t, $J = 7.0$ Hz, 6H, $2CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 163.52 and 161.66 (C=O, amide), 152.80 and 152.44 (C=O, ureide), 148.07 (C–NO_2), 144.64 (pyrazole C-3), 142.86 (pyrazole C-5), 120.18 (pyrazole C-4), 34.30 and 34.08 (NH$\underline{CH_2}$), 15.03 and 14.70 (CH_3), 138.95, 131.07, 130.65, 130.56, 130.09, 128.97, 127.22, 123.41, 119.60. Anal. calcd. for $C_{23}H_{23}N_7O_6$ (493.47 g/mol): C, 55.98; H, 4.70; N, 19.87. Found: C, 55.88; H, 4.74; N, 19.91.

*3.2.25. N^3,N^4-bis(ethylcarbamothioyl)-1-(3-nitrophenyl)-5-phenyl-1H-pyrazole-3,4-dicarbox amide (**25**)*

Synthesized from **4** (0.39 g, 1 mmol) and *N*-ethylthiourea (0.213 g, 2 mmol) according to the general procedure. The crude product was purified by crystallization from ethanol. Yield: 71%; m.p.: 242–244 °C; IR (ν, cm^{-1}): 3376 and 3237 (NH), 3036 (CH, aromatic), 2973 (CH, aliphatic), 1653 (C=O, ureide), 1602–1453 (C=C and C=N), 1343 (N–O sym.); ^{1}H NMR (500 MHz, DMSO-d_6) δ_H (ppm): 11.69 and 10.49 (s, 2H, 2NH), 10.46 (br, s, 2H, 2NH), 8.31–7.36 (m, 9H, ArH), 3.67 and 3.56 (pentet, J = 7.1 Hz, 4H, 2NH$\underline{CH_2}CH_3$), 1.21 and 1.14 (t, J = 7.1 Hz, 6H, $2CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C (ppm): 179.07 and 178.23 (C=S, thiourea), 162.58 and 159.81 (C=O, ureide), 147.91 (C–NO_2), 145.07 (pyrazole C-3), 142.18 (pyrazole C-5), 120.35 (pyrazole C-4), 39.57 (NH$\underline{CH_2}$), 13.25 (CH_3), 138.62, 131.40, 130.71, 130.16, 129.89, 128.91, 126.69, 123.62, 118.31. Anal. calcd. for $C_{23}H_{23}N_7O_4S_2$ (525.60 g/mol): C, 52.56; H, 4.41; N, 18.65; S, 12.20. Found: C, 52.43; H, 4.45; N, 18.66; S, 12.17.

3.3. In vitro antibacterial and antifungal activities of the molecules

In the current study, 5 bacteria and 5 yeast species were used as test organisms. Among these species, ATCC 10145, NRLL 3704, ATCC 25923, NRRL 12983 were utilized as standard strains. Vancomycine resistant *Enterococcus faecium*, Methicillin-resistant *S. aureus* (MRSA), *C. albicans*, *C. glabrata* and *C. tropicalis* (clinical isolates) were obtained from patients, and were provided by the Microbiology Laboratory of the Faculty of Medicine at the Eskisehir Osmangazi University, Turkey. *C. parapsilosis* (clinical isolates) was obtained from patients at the Microbiology Laboratory of Evliya Çelebi Hospital of the Faculty of Medicine, Kütahya Dumlupinar University, Turkey.

Bacterial and fungal cultures of test organisms were maintained on Nutrient Agar and Saboraud Dextrose Agar slants at 4 °C, respectively, and were sub-cultured in petri dishes prior to use. Antimicrobial activity analysis of test compounds was carried out according to modified agar well diffusion assay [40]. The compounds were dissolved first in Dimethylsulfoxide (DMSO, Merck) with the initial concentration of 2.5 mg/ml. Each solution was diluted two-fold with DMSO. These solutions were used in MIC test. Fifteen milliliters of the specified molten agar (45 °C) were poured into sterile Petri dishes. The cell suspensions containing 10^8 CFU/mL cells for bacteria, 10^7 CFU/mL cells for yeasts were prepared and evenly spread onto the surface of the agar plates of Nutrient Agar (Merck 1.05450) for bacteria and Saboraud Dextrose Agar (CM0041, Oxoid) medium for yeast using sterile swab sticks. The plates were dried aseptically at 35 °C for about 40 min in an incubator. The agar well method for the estimation of MIC values (the lowest concentration of compounds required to inhibit the growth of the tested microorganisms) was applied to evaluate the antimicrobial activity. At the same time, 6 mm wells were bored and 40 μl from each solution, diluted according to the two fold method previously, was placed into each well for MIC detection. The plates were pre-incubated for 2 h at 25 °C, followed by incubating the plates with bacterial strains 24 h at 37 °C, and the one with yeasts 48 h at 25 °C. Antimicrobial activity was evaluated as zones of inhibition of growth around wells. All samples were tested in duplicate. Cefotaxime (CTX; 30 μg/disc), for bacteria and nystatin (100 U/disc) for yeast were used as positive control.

4. Conclusions

The pyrazole derivatives studied in the current study depicted stronger inhibition effect on *C. albicans*. Among the Candida species tested, majority of the novel pyrazole derivatives were able to inhibit only the standard and the clinical *C. albicans* strains, while the other Candida species remained unaffected by the derivatives. However, only the molecules **8**, **10**, **21**, and **22** showed some inhibitory effects on *C. parapsilosis*, *C. tropicalis,* and *C. glabrata* strains. The structure-antifungal activity relationships of the compounds on the *C. albicans* strains investigated by using electron-conformational method (ECM). ECM study reveals that the estimation of the antifungal activity is not fully possible only with substituent-based considerations. In addition to geometric arrangements of some groups revealed by the present ECM study, their electronic parameters like the charges on atoms and the bond order values are also crucial for the intensity of the antifungal activity.

Among the presently studied molecules, the compounds **9,** and **21**–**25** are strongly antifungal on both the clinical isolate of *C. albicans* and the standard *C. albicans* strain, analogous to antifungal drugs amphotericin B, ampicillin, ciprofloxacin, fluconazole, streptomycin, nystatin, bifonazole, and miconazole [41–43]. In terms of MIC values, the presently studied pyrazole derivatives have comparable antifungal activities on *C. albicans* with the previously studied pyrazole derivatives of the class (1,4)-naphthoquinono-[3,2-c]-1H-pyrazoles, (1,4)-naphthohydroquinone-[3,2-c]-1H-pyrazoles, halogenated 4-[1H-imidazol-1-yl(phenyl) methyl]-1,5-diphenyl-1H-pyrazoles, pyrazole-1-carbothioamides, 1,3-disubstituted indeno[1,2-c]pyrazoles, and 4-arylidene pyrazoles [41–45]. However, they are more antifungal on *C. albicans* than the majority of pyrazoline and pyrazole derivatives with indoline and quinoxaline substituents [46]. Therefore, the present molecules constitute a new class of potent antifungal drugs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.033.

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