# One-pot synthesis and antiproliferative activity of highly functionalized pyrazole derivatives

Matteo Lusardi,[a] Chiara Rotolo,[a] Marco Ponassi,[b] Erika Iervasi,[b] Camillo Rosano,[b] and Andrea Spallarossa*[a]

[a] Dr. M. Lusardi, Dr. C. Rotolo, Prof. A. Spallarossa
Department of Pharmacy
University of Genova
viale Benedetto XV, 3, 16132, Genova, Italy
E-mail: andrea.spallarssa@unige.it
[b] Dr. M. Ponassi, Dr. E. Iervasi, Dr. C. Rosano
Proteomics and Mass Spectrometry Unit
IRCCS Ospedale Policlinico San Martino,
Largo R. Benzi 10, 16132, Genova, Italy

Supporting information for this article is given via a link at the end of the document

**Abstract:** A series of highly functionalized pyrazole derivatives has been prepared by a one-pot, versatile and regioselective procedure. Pyrazoles **1-29** were tested in cell-based assay to assess their antiproliferative activity against a panel of tumour cells. Additionally, the cytotoxicity of prepared compounds was evaluated against normal human fibroblasts. The antiproliferative activity of the synthesized molecules emerged to be affected by the nature of the substituents of the pyrazole scaffold and derivatives **21-23** proved to inhibit the growth of melanoma and cervical cancer cells. Compound **23** was identified as the most active derivative and docking simulations predicted its ability to interact with estrogen receptors.

## Introduction

Push-pull alkenes are substituted alkenes bearing one or two electron-donating groups at one end of the C=C bond and one or two electron-accepting groups at the other end. This arrangement promotes the π delocalization and the intramolecular charge transfer from the electron-donating groups ("push" terminus) to the electron-withdrawing groups ("pull" terminus) thus effecting both the molecular structure [e.g. central double bond elongation,[1] lowering of its rotational barrier][2,3] and the physical-chemical properties of the compounds [e.g. existence of strong charge-transfer absorption bands,[4] large dipole moments,[5] and high hyperpolarizabilities [6,7,8]]. As a number of theoretical and experimental investigations assessed,[9-28] the form that better describes a push-pull alkene is the zwitterion **D** (Figure 1), characterized by a strong charge transfer, large molecular dipole and quadrupole moments and, consequently, high polarization. These features make push-pull alkenes particularly reactive with nucleophilic and electrophilic species and therefore these compounds represent versatile building blocks for the synthesis of (hetero)cycles.

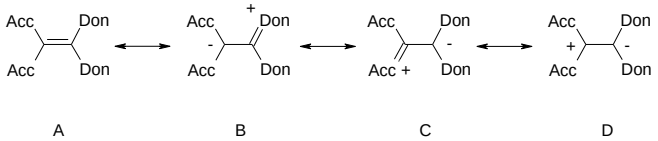



**Figure 1.** Different resonance structures for push-pull alkenes.

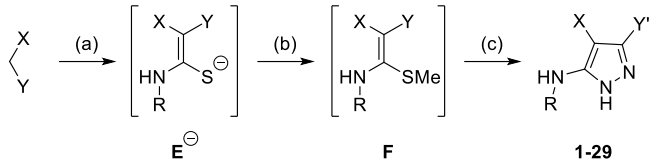
Functionalized pyrazoles and their fused analogues constitute the core structures of blockbuster drugs such as Acomplia, Celebrex and Viagra, as well as promising compounds endowed with different activities such as analgesic,[29] anti-inflammatory,[30] antipyretic,[31] antidepressant,[32] antibacterial,[33] antimicrobic,[34] and anti-cancer.[35] Within anti-tumour therapeutic area, a number of pyrazole derivatives proved to be potent and selective inhibitors of protein kinases, a class of enzyme that, through the phosphorylation of different substrates, plays a pivotal role in cell cycle.[36] Therefore, the chemical studies focused on both the development of novel procedures and the improvement of the existing protocols for the preparation and functionalization of pyrazoles are of certain interest in the field of medicinal chemistry.[37]

Owing to our interest in the development of diversity-oriented syntheses,[38] we focused our investigation on the reactivity of ketene aminothioacetals as representative structures of push-pull olefins (Scheme 1). The X and Y substituents represent the "pull"-terminus whereas the NHR and SMe groups constitute the "push" terminus. The push-pull intermediates were obtained by condensing active methylene reagents (AMRs) with isothiocyanates and were further modified leading to the formation of highly substituted pyrazoles **1-29** (Scheme 1). The developed synthetic methodology was based on both the synthetic and pharmaceutical attractiveness of the final procedure.

## Results and Discussion

*Chemistry*

The synthesis of pyrazoles **1-29** (Scheme 1) was carried out by a sequential, one-pot procedure. Briefly, the reaction between AMRs and isothiocyanates in the presence of sodium hydride in anhydrous DMF led to the corresponding N,S-ketene thioacetal salts $\mathbf{E}^-$ that were methylated "*in situ*" with iodomethane, affording the formation of the S-methyl, N-ketene acetal **F** (Scheme 1). These intermediates were then condensed "*in situ*" with anhydrous hydrazine to afford the regioselective formation of the desired 3,4,5-tri-substituted pyrazoles **1-29** (Scheme 1). The selected building blocks (Chart 1) were characterized by a high molecular diversity and include cyclic and linear AMRs as well as aliphatic and aromatic isothiocyanates.



**Scheme 1.** Synthesis of 3,4,5-trisubstituted pyrazoles; Reaction Conditions: (a) RNCS, NaH, DMF, rt. (b) MeI. (c) $NH_2NH_2$ anhydrous, 100 °C.

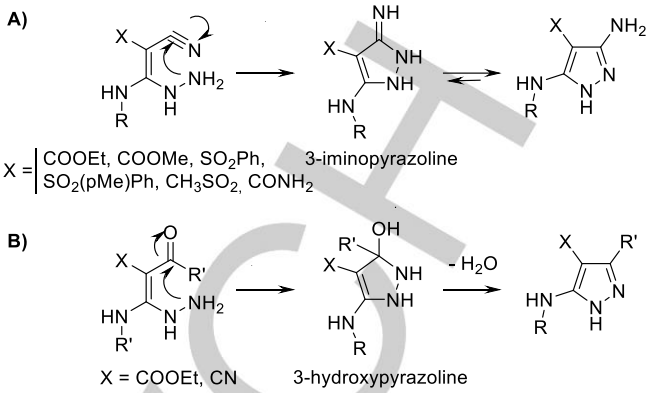
| X | Y |
|---|---|
| COOMe | CN |
| COOEt | CN |
| COOMe | COMe |
| COOEt | COPh |
| COOEt | CO(4-$NO_2$)Ph |
| $CONH_2$ | CN |
| CN | CN |
| CN | $COCMe_3$ |
| CN | COPh |
| CN | CO(3-Br)Ph |
| CN | CO(4-F)Ph |
| CN | CO(4-Cl)Ph |
| CN | CO(4-Br)Ph |
| CN | 2-thenoyl |
| COPh | COPh |
|  | |
| $SO_2Me$ | CN |
| $SO_2Ph$ | CN |
| $SO_2$(4-Me)Ph | CN |

| R−NCS |
|---|
| R |
| $C_6H_{11}$ |
| $CH_2Ph$ |
| Ph |
| 2-,3-,4-$NO_2$Ph |
| 2-,3-,4-OMePh |

**Chart 1.** Selected AMR and isothiocyanate building blocks

The combination of the selected building-blocks led to the preparation of a library of twenty-nine pyrazoles in overall yields ranging from 15% to 82 % (Table 1). The modular character of the synthesis allowed the independent variation of AMRs and isothiocyanates and therefore a wide variety of functional groups with different electronic and steric properties can be inserted at positions 3, 4 and 5 of the pyrazole ring. As reported in Table 1, some of the prepared pyrazoles were already described in literature.[39-49] However, none of them was obtained via a one pot procedure. In fact, the majority of the already published pyrazoles were synthesized through a 4-steps procedure which involved: i) the condensation of the proper AMRs with carbon disulfide, ii) the S-methylation of the corresponding ketene dithioacetal; iii) the displacement of the S-methyl group with the proper aniline; iv) the cyclization reaction with hydrazine.[39,40,41,43] Even in those cases where a similar reaction sequence was used (**10**, **14**, **20** and **26**) and/or the multi-step method led to higher pyrazole yields (**7**, **10**, **14**, **15**, **20** and **26**) our procedure represents a cheaper alternative either in terms of time (a unique final purification step is required) or cost (solid phase is more expensive).

As emerged by the $^1$H-NMR spectroscopic analysis of pyrazoles **1-29**, the adopted synthetic procedure was highly regioselective allowing the isolation of a unique pyrazole isomer. In particular, we observed that the hydrazinic amine group reacted selectively with X or Y, in the cyclization reaction (Scheme 2). Thus, when X = COOR and Y = CN, the hydrazinic amine group attacks the nitrile group (Scheme 2A), leading to the unique isolation of the 3-aminopyrazole derivative. Conversely, when X = CN or COOR and Y = COR, the cyclization reaction occurs on the ketone even in the presence of a pronounced steric hindrance (e.g. t-Bu). (Scheme 2B). These observations allowed to outline the following reactivity trend for the cyclization reaction: keto > cyano > ester.




**Scheme 2.** Regioselectivity and proposed mechanism for the cyclization reaction

*Antiproliferative assay*

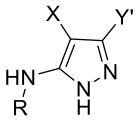
The antiproliferative properties of compounds **1-29** were assessed on a panel of tumour cell lines (breast cancer: MCF7, MDA-MB231, SK-BR3; melanoma: SKMEL-28; ovarian cancer: SKOV-3; liver cancer: Hep-G2; cervical cancer: HeLa; lung cancer: A549) by MTT assay (Table 2). Under the same conditions, the prepared pyrazoles were tested for their cytotoxicity in normal human fibroblasts GM-6114. Cisplatin was used as reference drug. All tested derivatives were assayed at a fixed concentration (10 μM). Pyrazoles **1-29** proved to be ineffective (growth percentage higher than 50%) against the proliferation of MCF7, MDA-MB231, SKOV-3, HepG2 and A549 cells. Conversely, derivatives **21-23**, sharing a 4-halosubstituted phenyl ring at position 3 and a nitrile group at position 4, showed significant antiproliferative activity against SKMEL-28 melanoma cell line with growth inhibition percentage values similar to that determined for cisplatin. Interestingly, this substitution pattern led to effective compounds against HeLa cells (derivatives **18**, **21-23**, Table 2), being pyrazole **23** the most active derivative (growth percentage 34.5%). Additionally, 3-phenyl-4-carboxyethyl pyrazole **12**, bearing a different substituent at position 4 (COOEt vs CN), showed similar antiproliferative properties to its 3-phenyl-4-cyano analogues against HeLa cells, thus indicating the relevance of the aromatic substituent on the heterocyclic core. Finally, derivative **23** was active against SK-BR3 breast cancer cell line. Differently from the reference compound, all the active derivatives against tumor cell lines were devoid of any cytotoxicity against normal fibroblasts GM-6114, supporting a specific mechanism of action towards mutated cells.

*Docking simulations*

The prevalent antiproliferative activity of pyrazole analogues **21-23** against SKMEL-28 and HeLa cells prompted us to evaluate the ability of these compounds to bind ER receptors. Interestingly, cervical cancer HeLa cell proliferation was increased by estradiol[50] thus demonstrating a relationship between estrogens and cell growth. Epidemiological data indicated that estrogens are involved in malignant melanoma as demonstrated by the higher survival rate in women with metastatic disease versus men and in premenopausal versus postmenopausal patients.[51] Moreover, both in vitro and in vivo

studies showed that estrogens directly influence malignant melanoma tumour growth,[52] especially through the binding of estrogen receptor beta, whose activation determines an antiproliferative effect.[53]

**Table 1.** List of key-intermediates **F** and pyrazoles **1-29**.




| Intermediate F | X | Y | Pyrazole cpd | Y' | R | Overall yield (%) One-pot | Overall yield (%) Stepwise[c] |
|---|---|---|---|---|---|---|---|
| **I** | COOMe | CN | **1** | $NH_2$ | $C_6H_{11}$ | 42 | |
| **II** | COOMe | CN | **2** | $NH_2$ | $CH_2Ph$ | 55 | |
| **III** | COOMe | CN | **3**[b] | $NH_2$ | Ph | 82 | 76[43] |
| **IV**[a] | COOMe | CN | **4** | $NH_2$ | (2-$NO_2$)Ph | 72 | |
| **V** | COOMe | CN | **5**[b] | $NH_2$ | (2-OMe)Ph | 65 | 55[43] |
| **VI** | COOMe | CN | **6** | $NH_2$ | (3-$NO_2$)Ph | 58 | |
| **VII** | COOMe | CN | **7**[b] | $NH_2$ | (3-OMe)Ph | 21 | 40[43] |
| **VIII** | COOMe | CN | **8** | $NH_2$ | (4-$NO_2$)Ph | 63 | |
| **IX** | COOMe | CN | **9**[b] | $NH_2$ | (4-OMe)Ph | 53 | 33[43] |
| **X** | COOEt | CN | **10**[b] | $NH_2$ | Ph | 37 | 53[46] |
| **XI**[a] | COOMe | COMe | **11**[b] | Me | Ph | 42 | NR[45] |
| **XII**[a] | COOEt | COPh | **12**[b] | Ph | Ph | 67 | NR[45] |
| **XIII**[a] | COOEt | CO(4-$NO_2$)Ph | **13**[b] | (4-$NO_2$)Ph | Ph | 30 | NR[45] |
| **XIV** | $CONH_2$ | CN | **14**[b] | $NH_2$ | Ph | 15 | 51[47] |
| **XV** | CN | CN | **15**[b] | $NH_2$ | Ph | 65 | 66[47] |
| **XVI** | CN | COt-Bu | **16** | t-Bu | Ph | 47 | |
| **XVII** | CN | COPh | **17**[b] | Ph | Ph | 72 | 48[48] |
| **XVIII**[a] | CN | CO(3-Br)Ph | **18** | (3-Br)Ph | Ph | 32 | |
| **XIX**[a] | CN | CO(4-F)Ph | **19** | (4-F)Ph | Ph | 33 | |
| **XX** | CN | CO(4-Cl)Ph | **20**[b] | (4-Cl)Ph | Ph | 68 | 98[39] |
| **XXI**[a] | CN | CO(4-Cl)Ph | **21** | (4-Cl)Ph | (4-$NO_2$)Ph | 63 | |
| **XXII**[a] | CN | CO(4-Cl)Ph | **22** | (4-Cl)Ph | (4-OMe)Ph | 54 | |
| **XXIII** | CN | CO(4-Br)Ph | **23** | (4-Br)Ph | Ph | 73 | |
| **XXIV** | CN |  | **24** |  | Ph | 79 | |
| **XXV** | COPh | COPh | **25** | Ph | Ph | 54 | |
| **XXVI** | |  | **26**[b] |  | Ph | 39 | 43[43] |
| **XXVII** | $SO_2Me$ | CN | **27** | $NH_2$ | Ph | 62 | |
| **XXVIII** | $SO_2Ph$ | CN | **28** | $NH_2$ | Ph | 73 | |
| **XXIX** | $SO_2$(4-Me)Ph | CN | **29**[b] | $NH_2$ | Ph | 76 | NR[49] |

[a] Unreported B intermediates. [b] Pyrazoles reported in literature, obtained in a stepwise fashion. [c] NR = not reported.

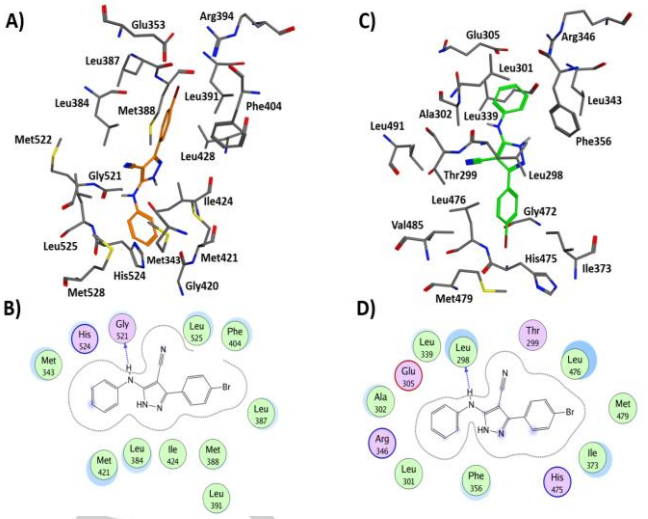



**Figure 2.** A) ERα-**23** docking complex. The ligand is coloured orange. B) Ligplot of ERα-**23** docking complex C) ERβ-**23** docking complex. The ligand is coloured green. D) Ligplot of ERβ-**23** docking complex.

Additionally, a number of tri- or tetra-substituted isoxazoles,[54] imidazoles,[54] furans,[55] thiophenes[56] and pyrazoles,[57] were identified as potent ER ligands. These derivatives are characterized by a central heteroaromatic scaffold diaryl-substituted at alternate positions. Similarly, compounds **21-23** bear the central pyrazole core substituted at position 3 and 5 with aromatic substructures. Supported by this evidence, docking simulations (Autodock4.2) were carried out to predict the binding mode of **23** (identified as the most promising compound of the series) in estrogen receptor (ER) alpha and beta binding sites. In the ERα-23 docking model, the ligand would assume an extended conformation with the exocyclic NH group forming a hydrogen bond with Gly521 carbonyl oxygen (Figures 2A,B). The complex would be further stabilized by van der Waals interactions between N-phenyl ring and Met343, Met421, Ile424, His524 side chain and Leu525 main chain. Additionally, the nitrile group would be in contact with Leu525 side chain and the pyrazole nitrogen atoms would interact with Met388, Ile424 and Gly521. Finally, the p-bromophenyl ring would be in contact with Leu387, Met388 and Phe404. The calculated Ki value for ERα-**23** complex is 5.5 μM.

The calculated Ki value for ERβ-**23** docking model is 7.5 μM, thus indicating a preferential binding of the ligand for ERα. Similarly to what has been predicted for ERα-**23** complex, the ERβ-**23** complex would be mainly stabilized by a hydrogen bond between the exocyclic NH group and Leu298 carbonyl oxygen (Figures 2C,D). Additional stabilization to the complex would be provided by the interactions between the N-phenyl ring and Leu301, Ala302, Glu305, Leu339, Arg346 and Phe356. Furthermore, the CN group would be in contact with Thr299, Ala302 and Leu476 and the pyrazole scaffold would interact with Leu298 and Phe356 side chains. The 4-bromophenyl system would interact with Ile373, His475, Leu476 and Met479 through van der Waals contacts.

**Table 2.** Antiproliferative activity of pyrazoles against different tumour cell lines.

| | Mean growth percentage[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cpd | MCF7 | MDA-MB231 | SK-BR3 | SKMEL28 | SKOV3 | Hep-G2 | A549 | HeLa | GM-6114 |
| **1** | 111.73 | 101.25 | 91.74 | 102.68 | 99.09 | 99.14 | 109.71 | 82.77 | 84.07 |
| **2** | 109.77 | 98.51 | 90.51 | 102.05 | 97.80 | 99.88 | 109.73 | 90.99 | 84.85 |
| **3** | 113.04 | 103.90 | 80.53 | 100.24 | 104.93 | 92.68 | 109.76 | 93.42 | 82.06 |
| **4** | 76.98 | 92.94 | 75.09 | 79.59 | 98.22 | 54.25 | 101.59 | 38.44 | 80.06 |
| **5** | 104.42 | 107.25 | 91.51 | 101.58 | 113.29 | 96.43 | 121.76 | 104.18 | 91.68 |
| **6** | 118.02 | 96.27 | 87.09 | 99.14 | 113.55 | 103.63 | 115.23 | 103.22 | 86.73 |
| **7** | 105.16 | 101.08 | 94.39 | 98.75 | 110.42 | 104.49 | 121.63 | 102.82 | 88.52 |
| **8** | 99.39 | 77.54 | 91.49 | 102.25 | 79.39 | 96.76 | 114.14 | 58.66 | 75.98 |
| **9** | 102.44 | 99.67 | 88.05 | 98.28 | 102.03 | 103.92 | 113.40 | 97.88 | 90.90 |
| **10** | 104.40 | 108.66 | 88.66 | 107.75 | 115.06 | 87.80 | 120.52 | 88.45 | 83.38 |
| **11** | 111.03 | 111.53 | 102.68 | 97.24 | 109.61 | 98.90 | 107.27 | 106.46 | 101.14 |
| **12** | 71.70 | 77.27 | 104.23 | 74.08 | 83.07 | 79.44 | 59.76 | 45.79 | 69.40 |
| **13** | 121.59 | 116.85 | 88.66 | 120.64 | 110.01 | 107.32 | 90.70 | 61.78 | 89.02 |
| **14** | 96.32 | 105.45 | 91.36 | 102.42 | 101.73 | 103.47 | 118.51 | 92.75 | 93.06 |
| **15** | 104.45 | 104.99 | 92.46 | 89.00 | 103.69 | 103.73 | 108.76 | 94.65 | 89.55 |
| **16** | 102.41 | 117.39 | 86.76 | 94.36 | 105.99 | 95.19 | 112.09 | 99.25 | 81.62 |
| **17** | 108.73 | 116.10 | 76.28 | 95.28 | 104.12 | 99.45 | 114.23 | 106.31 | 91.05 |
| **18** | 70.46 | 77.27 | 76.12 | 52.27 | 73.68 | 67.55 | 57.73 | 44.96 | 77.77 |
| **19** | 101.67 | 113.86 | 80.62 | 98.49 | 106.25 | 111.32 | 114.80 | 85.46 | 95.85 |
| **20** | 71.13 | 90.80 | 64.98 | 71.71 | 62.11 | 102.37 | 61.03 | 77.10 | 94.91 |
| **21** | 79.33 | 61.45 | 79.83 | 46.94 | 67.66 | 54.37 | 55.33 | 43.57 | 62.98 |
| **22** | 88.35 | 72.41 | 55.91 | 44.36 | 69.32 | 54.13 | 52.44 | 43.99 | 69.62 |
| **23** | 60.80 | 63.69 | 45.54 | 43.75 | 53.51 | 51.81 | 52.01 | 34.51 | 66.65 |
| **24** | 99.74 | 106.06 | 77.86 | 94.19 | 98.12 | 104.77 | 111.75 | 94.35 | 85.35 |
| **25** | 117.65 | 120.40 | 99.30 | 114.61 | 115.31 | 113.18 | 106.46 | 100.84 | 84.10 |
| **26** | 85.23 | 84.12 | 80.82 | 53.57 | 80.26 | 58.86 | 59.85 | 68.30 | 82.47 |
| **27** | 97.61 | 105.07 | 93.60 | 106.18 | 104.10 | 106.24 | 115.07 | 98.83 | 89.36 |
| **28** | 110.32 | 115.27 | 96.36 | 106.61 | 117.57 | 110.81 | 122.08 | 85.04 | 95.85 |
| **29** | 115.17 | 117.02 | 96.05 | 112.07 | 118.49 | 110.26 | 126.76 | 95.92 | 94.00 |
| **Cisplatin** | 72.74 | 86.07 | 70.59 | 44.40 | 36.83 | 38.07 | 59.09 | 29.33 | 39.52 |

[a] Data mean values for three separate experiments. Variation among triplicate samples was less than 10%

.

## Conclusion

A series of highly substituted pyrazole derivatives has been synthesized through an unprecedented one-pot, regioselective procedure. The developed synthetic method proved to be versatile as demonstrated by the different substituents decorating the pyrazole scaffold of derivatives **1-29**. The prepared compounds were tested in cell-based assay for their cytotoxicity against a panel of nine cell line. Derivatives **21-23** emerged to be specifically active against SKMEL-28 (melanoma) and HeLa (cervical cancer) cells without altering the growth of normal fibroblasts GM-6114. As melanoma and cervical cancer are estrogen dependent tumours and active compounds **21-23** share common chemical feature with estrogen receptor ligands, docking simulations were carried out to assess the binding affinity of **23** (the most active derivative of the series) towards ERα and ERβ. The computational results predicted for **23** micromolar Ki values for both receptors, thus indicating ER as a potential target for the activity of this class of pyrazoles. On this basis, future studies will aim to validate the docking results and further develop SARs to increase the antiproliferative activity of this series of derivatives.

## Experimental Section

*Chemistry*

Commercially available active methylene reagents, isothiocyanates, hydrazine hydrate and reagents (55% sodium hydride dispersion in mineral oil, iodomethane) were purchased by Alfa-Aesar and Sigma-Aldrich. 4-Bromobenzoylacetonitrile, 3-

bromobenzoylacetonitrile, 4-fluorobenzoylacetonitrile were prepared according to the published procedure.[58] DMF was reagent grade and was dried on molecular sieves (5Å 1/16" inch pellets). Unless otherwise stated, all commercial reagents were used without further purification. Organic solutions were dried over anhydrous sodium sulphate. Thin layer chromatography (TLC) system for routine monitoring the course of parallel reactions and confirming the purity of analytical samples employed aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F254). Neat DCM or DCM/methanol (9:1) mixture were used as a developing solvent and detection of spots was made by UV light and/or by iodine vapors. The parallel solution phase chemistry was performed by using a 12-Carousel Reaction Station™ (Radleys Discovery Technologies, Italian distributor: StepBio, Bologna). The evaporation of solutions in parallel fashion was performed with an Evaposel™ apparatus (Radleys Discovery Technologies, Italian distributor: StepBio, Bologna) operating at reduced pressure of about 15-20 Torr. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Varian Gemini or JEOL JNM-ECZR instrument; chemical shifts were reported in δ (ppm) units relative to the internal reference tetramethylsilane, and the splitting patterns were described as follows: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). The first order values reported for coupling constants *J* were given in Hz. Elemental analyses were performed by an EA1110 Analyzer, Fison Instruments (Milan).

**General parallel synthetic procedure for the preparation of Pyrazoles 1-29.**

In each reaction tube of a Carusel Reaction Station™ a solution of the active methylene reagent (10 mmol) in dry DMF (10 ml) was poured and a 55% sodium hydride dispersion in mineral oil (10 mmol) was added under stirring at rt. After 45 minutes the proper isothiocyanate (10 mmol) was added in a single portion. Each reaction mixture was stirred for 1 h at rt, then iodomethane (10 mmol) was added. In many cases a gelatinous precipitate was observed. After 3 hs, the reaction mixture was treated with freshly distilled anhydrous hydrazine (25 mmol) at rt and heated at 95-100°C for 4 hs. Each reaction was transferred into a set of separating funnels, diluted with water (150 mL) and extracted with ether or dichloromethane (30 mL x 3). The combined extracts were washed with water (30 mL x 5), dried and filtered. Evaporating in vacuo gave a residue that was purified by crystallization from the suitable solvent or solvent mixture.

*Methyl 3-amino-5-(cyclohexylamino)-1H-pyrazole-4-carboxylate (1).* Mp 120-122°C (Ether-Ligroin 1:1); Yield: 42%. $^1$H NMR (400 MHz, DMSO-d6): δ 1.13-1.91 (m, 10H, cyclohexyl.); 3.65 (s, 3H, $CH_3O$); 4.97 (bs, 1H, NH phenyl., exchangeable); 5.80 (bs, 2H, $NH_2$, exchangeable); 10.51 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 164.67; 154.04; 150.86; 81.01; 50.36; 49.92; 32.84; 25.36; 24.44. Calcd for $C_{11}H_{18}N_4O_2$ : C= 55.45; N= 23.51; H= 7.61. Found: C= 55.55; N= 22.44; H= 7.60.

*Methyl 3-amino-5-(benzylamino)-1H-pyrazole-4-carboxylate (2).* Mp 173-175°C (Ether); Yield: 55%. $^1$H NMR (400 MHz, DMSO-d6): δ 3.66 (s, 3H, $CH_3O$); 4.33 (d, 2H, $CH_2$); 5.85 (bs, 2H, $NH_2$, exchangeable); 7.22-7.32 (m, 5H, arom. H + NH phenyl., exchangeable); 10.58 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 164.60; 151.00; 128.17; 127.16; 126.55; 81.12; 49.93; 45.67. Calcd for $C_{12}H_{14}N_4O_2$: C= 58.53; N= 22.75; H= 5.73. Found: C= 58.32; N= 23.07; H= 5.94.

*Methyl 3-amino-5-anilino-1H-pyrazole-4-carboxylate (3).* Mp 191-193°C (DCM-MeOH 2:1); Yield: 82%. $^1$H NMR (400 MHz, DMSO-d6): δ 3.75 (s, 3H, $CH_3O$); 6.07 (bs, 2H, $NH_2$, exchangeable); 6.79-6.82 and 7.20-7.24 and 7.53-7.55 (m, 5H, arom. H); 7.99 (bs, 1H, NH phenyl., exchangeable); 11.04 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 164.71; 150.78; 150.15; 141.59; 128.70; 119.34; 116.21; 81.60; 50.33. Calcd for $C_{11}H_{12}N_4O_2$: C= 56.89; N= 24.12; H= 5.21. Found: C= 57.26; N= 23.80; H= 5.63.

*Methyl 3-amino-5-[(2-nitrophenyl)amino]-1H-pyrazole-4-carboxylate (4).* Mp 294-296°C (DCM-MeOH 2:1); Yield: 72%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.82 (s, 3H, $CH_3O$); 6.25 (bs, 2H, $NH_2$, exchangeable); 7.00-7.67 and 8.21-8.92 (m, 4H, arom. H), 11.12 (bs, 1H, NH phenyl., exchangeable); 11.50 (bs, 1H, NH pyraz., exchangeable.). Calcd for $C_{11}H_{11}N_5O_4$: C= 47.66; N= 25.26; H= 4.00. Found: C= 47.64; N=24.89; H=4.66.

*Methyl 3-amino-5-[(2-methoxyphenyl)amino]-1H-pyrazole-4-carboxylate (5).* Mp 226-228°C (DCM-MeOH 2:1); Yield: 65%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.75 (s, 3H, $CH_3O$); 3.88 (s, 3H, $CH_3O$ phenyl.); 6.07 (bs, 2H, $NH_2$, exchangeable); 6.70-7.00 and 8.19-8.22 (m, 4H, arom. H); 8.51 (bs, 1H, NH phenyl., exchangeable); 11.06 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{12}H_{14}N_4O_3$: C= 54.96; N= 21.36; H= 5.38. Found: C= 54.81; N=20.98; H=5.24.

*Methyl 3-amino-5-[(3-nitrophenyl)amino]-1H-pyrazole-4-carboxylate (6).* Mp 271-273°C (DCM-MeOH 2:1); Yield: 58%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.78 (s, 3H, $CH_3O$); 6.21 (bs, 2H, $NH_2$, exchangeable); 7.42-7.78 (m, 4H, arom. H); 8.85 (bs, 1H, NH phenyl., exchangeable); 11.05 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{11}H_{11}N_5O_4$: C= 47.66; N= 25.26; H= 4.00. Found: C= 47.82; N=24.76; H=3.70.

*Methyl 3-amino-5-[(3-methoxyphenyl)amino]-1H-pyrazole-4-carboxylate (7).* Mp 184-186°C (DCM-MeOH 2:1); Yield: 21%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.73 (s, 3H, $CH_3O$); 3.74 (s, 3H, $CH_3O$ phenyl.); 6.09 (bs, 2H, $NH_2$, exchangeable); 6.32-6.45 and 6.93-7.19 and 7.28-7.37 (m, 4H, arom. H); 8.01 (bs, 1H, NH phenyl., exchangeable); 11.08 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{12}H_{14}N_4O_3$: C= 54.96; N= 21.36; H= 5.38. Found: C=54.71; N=21.13; H=5.36.

*Methyl 3-amino-5-[(4-nitrophenyl)amino]-1H-pyrazole-4-carboxylate (8).* Mp 280-282°C (DCM-MeOH 2:1); Yield: 63%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.77 (s, 3H, $CH_3O$); 6.23 (bs, 2H, $NH_2$, exchangeable); 7.70-7.85 and 8.10-8.8.22 (m, 4H, arom. H); 8.75 (bs, 1H, NH phenyl., exchangeable); 11.41 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{11}H_{11}N_5O_4$: C= 47.66; N= 25.26; H= 4.00. Found: C= 48.00; N=24.96; H= 4.36.

*Methyl 3-amino-5-[(4-methoxyphenyl)amino]-1H-pyrazole-4-carboxylate (9).* Mp 136-138°C (Ether-DCM 1:1); Yield: 53%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.70 (s, 3H, $CH_3O$); 3.80 (s, 3H, $CH_3O$ phenyl.); 6.08 (bs, 2H, $NH_2$, exchangeable); 6.70-7.05 and 7.35-7.70 (m, 4H, arom. H); 7.88 (bs, 1H, NH phenyl., exchangeable); 11.00 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{12}H_{14}N_4O_3$: C= 54.96; N= 21.36; H= 5.38. Found: C= 54.90; N=21.45; H=5.62.

*Ethyl 3-amino-5-anilino-1H-pyrazole-4-carboxylate (10).* Mp 170-172°C (DCM-MeOH 2:1) (Litt.:178°C-MeOH); Yield: 37%. $^1$H NMR (200 MHz, DMSO-d6): δ 1.32 (t, J = 7.8 Hz, 3H, $CH_3$); 4.30 (q, J = 7 Hz, 2H, $CH_2O$); 6,02 (bs, 2H, $NH_2$, exchangeable); 6.61-8.27 (m, 6H, arom. H + NH phenyl.); 11.40 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{12}H_{14}N_4O_2$: C= 58.53; N= 22.75; H= 5.73. Found: 58.53; N= 22.69; H= 6.10.

*Methyl 5-anilino-3-methyl-1H-pyrazole-4-carboxylate (11).* Mp 191-193°C (Ether); Yield: 42%. $^1$H NMR (400 MHz, DMSO-d6): δ 2.39 (s, 3H, $CH_3$); 3.79 (s, 3H, $CH_3O$); 6.82-6.85 and 7.23-7.27 and 7.56-7.58 (m, 5H, arom. H); 8.19 (bs, 1H, NH phenyl., exchangeable); 12.47 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 165.18; 152.86; 142.71; 141.47; 128.81; 119.62; 116.27; 95.75; 50.96; 11.57. Calcd for $C_{12}H_{13}N_3O_2$: C=62.33; N=18.17; H=5.67. Found: C=61.97; N=18.25; H=5.41.

*Ethyl 5-anilino-3-phenyl-1H-pyrazole-4-carboxylate (12).* Mp 155-157°C (DCM-MeOH 2:1); Yield: 67%. $^1$H NMR (400 MHz, DMSO-d6): δ 1.13 (t, J=7.1 Hz, 3H, $CH_3$); 4.16 (q, J= 7.1 Hz, 2H, $CH_2O$); 6.85-6.89 and 7.26-7.30 and 7.48-7.63 (m, 10H, arom. H); 8.45 (bs, 1H, NH phenyl., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 164.49; 153.49; 144.74; 141.44; 129.45; 129.32; 128.90; 127.94; 119.80; 116.37; 95.23; 59.63; 13.83. Calcd for $C_{18}H_{17}N_3O_2$: C=70.34; N=13.67; H= 5.58. Found: C=70.57; N=13.48; H=5.87.

*Ethyl 5-anilino-3-(4-nitrophenyl)-1H-pyrazole-4-carboxylate (13)*. Mp 165-167°C (DCM-MeOH 2:1); Yield: 30%. $^1$H NMR (400 MHz, DMSO-d6): δ 1.15 (t, J= 7.1 Hz, 3H, $CH_3$); 4.18 (q, J= 7.1 Hz, 2H, $CH_2O$); 6.86-6.90 and 7.27-7.31 and 7.62-7.92 and 8.33-8.35 (m, 9H, arom. H) 8.43 (bs, 1H, NH phenyl., exchangeable); 12.48 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 164.08; 153.68; 147.74; 142.45; 141.27; 135.16; 130.90; 128.92; 123.04; 120.01; 116.49; 96.00; 59.97; 13.79. Calcd for $C_{18}H_{16}N_4O_4$: C=61.36; N=15.90; H=4.58. Found: C=61.21; N=15.87; H=4.80.

*3-amino-5-anilino-1-pyrazole-4-carboxamide (14).* Mp 212-214°C (DCM-MeOH 2:1) (Litt.: 178°C-EtOH); Yield: 15%. $^1$H NMR (400 MHz, DMSO-d6): δ 5.84 (bs, 2H, $NH_2$, exchangeable); 6.69 (bs, 2H, $NH_2$ amide, exchangeable); 6.72-6.76 and 7.16-7.34 (m, 5H, arom. H); 8.99 (bs, 1H, NH phenyl., exchangeable); 10.99 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 166.65; 151.16; 147.73; 142.89; 128.73; 118.61; 115.69; 86.06. Calcd for $C_{10}H_{11}N_5O$: C= 55.29; N= 32.24; H= 5.10. Found: C=55.21; N=32.09; H=5.34.

*3-amino-5-anilino-1H-pyrazole-4-carbonitrile (15).* Mp 209-211°C (DCM-MeOH 2:1) (Litt.: 194-195°C-EtOH); Yield: 65%. $^1$H NMR (200 MHz, DMSO-d6): δ 6.25 (bs, 2H, $NH_2$, exchangeable); 6.73-7.14 and 7.41-7.44 (m, 5H, arom. H); 8.31 (bs, 1H, NH phenyl., exchangeable); 11.12 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{10}H_9N_5$: C= 60.29; N= 35.15; H= 4.55. Found: C= 59.96; N= 35.03; H= 4.90.

*5-anilino-3-tert-butyl-1H-pyrazole-4-carbonitrile (16).* Mp 183-185°C (Ether-DCM 1:1); Yield: 47%. $^1$H NMR (400 MHz, DMSO-d6): δ 1.38 (s, 9H, t-Bu); 6.75-6.83 (m, 1H, arom. H); 7.16-7.24 (m, 2H, arom. H); 7.43-7.50 (m, 2H, arom. H); 8.56 (bs., 1H, NH phenyl., exchangeable); 12.60 (bs., 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 29.28; 32.64; 77.12; 115.73; 116.66; 119.97; 129.12; 143.06; 154.11; 157.53. Calcd for $C_{14}H_{16}N_4$: C= 69.97; N= 23.31; H= 6.71. Found: C= 70.10; N= 23.23; H= 7.01.

*5-anilino-3-phenyl-1H-pyrazole-4-carbonitrile (17).* Mp 219-221°C (DCM-Ligroin 1:1); Yield: 72%. $^1$H NMR (400 MHz, DMSO-d6): δ 6.80-6.87 (m, 1H, arom. H); 7.20-7.29 (m, 2H, arom. H); 7.48-7.63 (m, 5H, arom. H); 7.79-7.86 (m, 2H, arom. H); 8.85 (bs, 1H, NH phenyl., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 77.14; 115.05; 116.38; 119.78; 126.40; 126.79; 128.71; 129.39; 130.27; 142.31; 146.39; 153.89. Calcd for $C_{16}H_{12}N_4$: C= 73.83; N= 21.52; H= 4.65. Found: 74.16; N= 21.76; H= 4.70.

*5-anilino-3-(3-bromophenyl)-1H-pyrazole-4-carbonitrile (18).* Mp 240-242°C (Ether-MeOH 2:1); Yield: 32%. $^1$H NMR (200 MHz, DMSO-d6): δ 6.58-8.28 (m, 9H, arom. H); 9.00 (bs, 1H, NH phenyl., exchangeable); 14.65 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{16}H_{11}BrN_4$: C= 56.66; N= 16.52; H= 3.27. Found: C= 56.57; N= 16.59; H= 3.61.

*5-anilino-3-(4-fluorophenyl)-1H-pyrazole-4-carbonitrile (19).* Mp 229-231°C (DCM-MeOH 2:1); Yield: 33%. $^1$H NMR (200 MHz, DMSO-d6): δ 6.76-8.34 (m., 9H, arom. H); 8.98 (bs, 1H, NH phenyl., exchangeable); 14.25 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{16}H_{11}FN_4$: C= 69.06; N= 20.13; H= 3.98. Found: C= 69.00; N= 19.76; H= 3.98.

*5-anilino-3-(4-chlorophenyl)-1H-pyrazole-4-carbonitrile (20).* Mp 240-242°C (DCM-MeOH 2:1); Yield: 68%. $^1$H NMR (400 MHz, DMSO-d6): δ 6.82-6.86 and 7.22-7.30 and 7.52-7.85 (m, 9H, arom. H); 8.85 (bs, 1H, NH phenyl., exchangeable); 9.23 (bs, 1H NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 153.93; 145.17; 142.21; 134.89; 129.47; 128.67; 128.11; 125.60; 119.82; 116.41; 114.81; 77.34. Calcd for $C_{16}H_{11}ClN_4$: C= 65.20; N= 19.01; H= 3.76. Found: C= 64.93; N= 19.29; H= 3.88.

*3-(4-chlorophenyl)-5-[(4-nitrophenyl)amino]-1H-pyrazole-4-carbonitrile (21).* Mp >300°C (Ether-Ligroin 1:1); Yield: 63%. $^1$H NMR (400 MHz, DMSO-d6): δ 7.56-7.73 (m, 4H, arom. H); 7.81-7.89 (m, 2H, arom. H); 8.13-8.21 (m, 2H, arom. H); 9.92 (bs, 1H, H phenyl., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 84.35; 119.49; 120.62; 130.81; 133.51; 134.78; 140.38; 144.61; 153.86. Calcd for $C_{16}H_{10}ClN_5O_2$: C= 56.57; N= 20.61; H= 2.97. Found: C= 56.34; N= 20.73; H= 3.11.

*3-(4-chlorophenyl)-5-[(4-methoxyphenyl)amino]-1H-pyrazole-4-carbonitrile (22).* Mp 272-274°C (DCM-MeOH 2:1); Yield: 54%. $^1$H NMR (200 MHz, DMSO-d6): δ 3.73 (s, 3H, $CH_3O$); 6.70-7.90 (m, 8H, arom. H); 8.70 (bs, 1H, NH phenyl., exchangeable); 13.23 (bs, 1H, H pyraz., exchangeable). Calcd for $C_{17}H_{13}ClN_4O$: C= 62.87; N=17.25; H= 4.03. Found: C= 63.06; N= 17.41; H= 4.24.

*5-anilino-3-(4-bromophenyl)-1H-pyrazole-4-carbonitrile (23).* Mp 256-258°C (Ether-MeOH 2:1); Yield: 73%. $^1$H NMR (200 MHz, DMSO-d6): δ 6.78-8.20 (m., 9H, arom. H); 8.95 (bs, 1H, NH phenyl., exchangeable); 14.55 (bs, 1H, NH pyraz., exchangeable). Calcd for $C_{16}H_{11}BrN_4$: C= 56.66; N= 16.52; H= 3.27. Found: C= 56.55; N= 16.32; H= 3.57.

*5-anilino-3-thien-2-yl-1H-pyrazole-4-carbonitrile (24).* Mp 243-245°C (Ether); Yield: 79%. $^1$H NMR (400 MHz, DMSO-d6): δ 6.89-7.10 and 7.24-7.79 (m, 8H, arom. H); 8.86 (bs, 1H, NH phenyl., exchangeable); 9.20 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 153.52;

141.28; 128.89; 128.14; 127.41; 121.65; 119.89; 116.64; 114.61; 76.87. Calcd for $C_{14}H_{10}N_4S$: C= 63.14; N= 21.04; H= 3.78; S= 12.04. Found: C= 63.02; N= 21.23; H= 3.93; S= 11.76.

*(5-anilino-3-phenyl-1H-pyrazol-4-yl)(phenyl)methanone (25).* Mp 196-198°C (Ether-DCM 1:1); Yield: 54%. $^1$H NMR (400 MHz, DMSO-d6): δ 6.86-6.92 (m, 1H, arom. H); 7.03-7.10 (m, 2H, arom. H); 7.11-7.18 (m, 4H, arom. H); 7.22-7.37 (m, 6H, arom. H); 7.64-7.71 (m, 2H, arom. H); 9.07 (bs, 1H, NH phenyl., exchangeable); $^{13}$C NMR (101 MHz, DMSO-d6): δ 105.04; 117.21; 120.51; 128.03; 128.51; 129.05; 129.28; 129.31; 129.48; 129.64; 131.58; 139.55; 141.91; 146.31; 154.44; 192.57. Calcd for $C_{22}H_{17}N_3O$: C= 77.86; N= 12.38; H= 5.05. Found: C= 77.86; N= 12.60; H= 4.97.

*3-anilino-6,6-dimethyl-2,5,6,7-tetrahydro-4H-indazol-4-one (26).* Mp 275-277°C (EtOH) (Litt.: 274-277°C-EtOH); Yield: 39%. $^1$H NMR (400 MHz, DMSO-d6): δ 1.05 (s, 6H, $CH_3$); 2.29 (s, 2H, $CH_2$); 2.68 (s, 2H, $CH_2$); 6.83-6.87 and 7.23-7.27 and 7.59-7.61 (m, 5H, arom. H); 7.94 (bs, 1H, NH phenyl., exchangeable); 12.45 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 192.60; 150.00; 148.76; 141.31; 128.79; 119.85; 116.37; 103.87; 51.30; 35.71; 34.06; 27.93. Calcd for $C_{15}H_{17}N_3O$: C= 70.56; N= 16.46; H= 6.71. Found: C=70.27; N= 16.41; H= 7.10.

*4-(methylsulfonyl)-N$^5$-phenyl-1H-pyrazole-3,5-diamine (27).* Mp 181-183°C (DCM-MeOH 2:1); Yield: 76%. $^1$H NMR (400 MHz, DMSO-d6): δ 3.11 (s, 3H, $CH_3$); 5.93 (bs, 2H, $NH_2$, exchangeable); 6.80-6.83 and 7.20-7.24 and 7.47-7.49 (m, 5H, arom. H); 7.39 (bs, 1H, NH phenyl., exchangeable); 11.31 (bs, 1H NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): δ 148.69; 147.69; 141.57; 128.69; 119.60; 116.39; 86.91; 44.82. Calcd for $C_{13}H_{18}N_4O_2S$: C= 47.61; N= 22.21; H= 4.79; S= 12.71. Found: C= 47.71; N= 22.43; H= 4.84; S=12.49.

*N$^5$-phenyl-4-(phenylsulfonyl)-1H-pyrazole-3,5-diamine (28).* Mp 182-184°C (Ether-DCM 1:1); Yield: 72%. $^1$H NMR (400 MHz, DMSO-d6): δ 6.14 (bs, 2H, $NH_2$, exchangeable); 6.82-6.86 and 7.20-7.24 and 7.51-7.64 and 7.97-7.98 (m, 10H, arom. H); 7.46 (bs, 1H, NH phenyl., exchangeable); 11.32 (bs, 1H, NH pyraz., exchangeable). $^{13}$C NMR (101 MHz, DMSO-d6): 148.69; 147.51; 144.15; 141.31; 132.78; 129.39; 128.72; 125.26; 119.91; 116.63; 86.84. Calcd for $C_{15}H_{14}N_4O_2S$: C= 57.31; N= 17.82; H= 4.49; S= 10.20. Found: C= 57.19; N= 18.03; H= 4.36; S= 10.09.

*4-[(4-methylphenyl)sulfonyl]-N$^5$-phenyl-1H-pyrazole-3,5-diamine (29).* Mp 170-172°C (Ether); Yield: 76%. $^1$H NMR (200 MHz, DMSO-d6): δ 2.30 (s, 3H, $CH_3$); 6.15 (bs, 2H, $NH_2$, exchangeable); 6.72-8.17 (m, 10H, arom. H + NH phenyl., exchangeable); 11.28 (bs, 1H, NH pyraz., exchangeable). Calcd for C16H16N4O2S: C= 58.52; N= 17.06; H= 4.91; S= 9.76. Found: C= 58.33; N= 16.82; H= 4.82; S= 9.38.

*Biology*

MTT assay was performed using SKOV-3 (ovarian adenocarcinoma, ATCC, Manassas, VA); MCF-7 (breast adenocarcinoma, Biologic Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy); Hep-G2 (hepatocellular carcinoma, ATCC, Manassas, VA); SK-MEL28 (skin melanoma, Biologic Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy), GM-6114 (embryonic human fibroblast, ATCC, Manassas, VA); MDA-MB231 (breast adenocarcinoma, Biologic Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy); HeLa (cervical adenocarcinoma, Biologic Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy); SK-BR3 (breast andenocarcinoma, Biologic Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy); A549 (lung carcinoma, Biologic Bank and Cell Factory, IRCCS Policlinico San Martino, Genoa, Italy) cell lines. All cell lines were grown in DMEM (with 10% FBS, 2 mM Glutamine and 1% penstrep. All reagents were purchased from EuroClone, Milan, Italy) and incubated at 37 °C in 5% $CO_2$ in a humidified environment. Briefly, all cell lines were plated in 96 well plates at an adequate number to reach 80%–90% of confluence at the end of the assay. 16 h after cell plating, compounds were dissolved in DMSO to give a 10 mM stock solution, diluted in growth medium and added at a final working concentration of 10 μM. After 48 h of incubation, 30 μL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) at a concentration of 2 mg/mL in PBS, were added in each well. Then, after further 4 h of incubation, the surnatant was removed and 100 μL/well of DMSO were used to dissolve the Formazan precipitate that can be found in vital cells. After 20 min, the results were read at 570 nm by means of a spectrophotometer. The results are expressed as percentage of the control samples in which the cells were incubated with the same amount of DMSO but without compounds. The assays were repeated three times. In each set, every single compound was tested six times. Means and standard deviations were calculated.

*Docking simulations*

The molecular structures of compound 23 was built by MOE2009.10 (builder module), parametrized by MMFF94x forcefield and its docking poses within ERα and ERb were calculated by Autodock 4.2.[59] After the removal of the water molecules and of the co-crystallized ligands, polar hydrogen and Gasteiger-Huckel charges were added to the crystal structures of ER alpha (PDB code 5TLD)[60] and beta (PDB code: 2YLY).[61] The ligands root was defined automatically. A 60 × 60 × 60 Å grid (grid spacing 0.375 Å) was centered in the binding site of the ligands and electrostatic and affinity maps for each atom type of the ligand were calculated. The docking search was performed over 100 conformers using the Genetic Algorithm Local Search protocol as implemented in Autodock (population size: 50; rate of gene mutation: 0.02; rate of crossover: 0.8). The docking poses were clustered (rmsd: 2.0 Å) and the best conformation of the low energy highest populated cluster was selected as the binding conformation. Models analysis was carried out using the CCP4 program suite.[62] The calculations were run on a Linux PC (Intel® processor Core™ i7-2600 CPU@3.40 GHz).

## Acknowledgements

Financial support was provided by University of Genova (FRA grant). The work by C.R. has been supported by the Italian Ministry of Health (Ricerca Corrente). C.R. acknowledge the support by the EU COST Action CA 17104 "STRATAGEM" (New diagnostic and therapeutic tools against multidrug resistant tumours).

**Keywords:** one-pot synthesis • pyrazole derivatives • antiproliferative agents • docking simulations

[1] D. Adhikesavalu, K. Venkatesan, *Acta Crystallgr.* **1983**, *39*, 1024-1426.

[2] G. Fischer, W.D. Rudorf, E. Kleinpeter, *Magn. Reson. Chem.* **1991**, *29*, 212-222.

[3] G. Fischer and E. Kleinpeter, *Magn. Reson. Chem.* **1991**, *29*, 204-206.

[4] S. Inoue, Y. Aso, T. Otsubo, *Chem. Commun.* **1997**, *12*, 1105-1106.

[5] B.A. Trofimov, V.B. Modonov, T.N. Aksamentova, A.I. Mikhaleva, S.E. Korostova, L.N. Sobenina, R.N. Nesterenko, R.I. Polovnikova, *Izv. Akad. Nauk. SSSR, Ser. Khim.* **1987**, *6*, 1322-1326.

[6] L.R. Dalton, A.W. Harper, R. Ghosn, W.H. Steier, M. Ziari, H. Fetterman, Y. Shi, R.V. Mustacich, A.K.Y. Jen, K.J. Shea, *Chem. Mater.* **1995**, *7*, 1060-1081.

[7] D.R. Kanis, M.A. Ratner, T.J. Marks, *Chem. Rev.* **1994**, *94*, 195-242.

[8] P.N. Prasad, D.J. Williams, in *Introduction to Nonlinear Optical Effects in Molecules and Polymers*, *Wiley*, New York USA **1991**.

[9] P. Rattananakin, C.U. Pittman, W.E. Collier, S. Saebo, *Struct. Chem.* **2007**, *18*, 399-407.

[10] E. Kleinpeter, *J. Serb. Chem. Soc.* **2006,** *71*, 1-17.

[11] E. Kleinpeter, A. Schulenburg, *Tetrahedron Lett.* **2005,** *46*, 5995-5997.

[12] E. Kleinpeter, S. Klod, W.D. Rudorf, *J. Org. Chem.* **2004,** *69*, 4317-4329.

[13] R. Markovic, A. Shirazi, Z. Dzambaski, M. Baranac, D. Minic, *J. Serb. Chem. Soc.* **2003**, *68*, 1-7.

[14] A. Forni, R. Destro, *Chem. Eur. J.* **2003,** *9*, 5528-5537.

[15] M.H. Matus, R. Contreras, A. Cedillo, M. Galvan, *J. Chem. Phys.* **2003,** *119*, 4112-4116.

[16] E. Stankovic, S. Toma, R. Van Boxel, I. Asselberghs, A. Persoons, *J. Organomet. Chem.* **2001,** *637*, 426-434.

[17] I. Yavari, N. Hazeri, M.T. Maghsoodlou, A. Moradi, *J. Chem. Res., Synop.* **2001,** *7*, 272-273.

[18] C. Orlewska, R.M. Shaker, M. Dees, H.H. Otto, *Monatsh. Chem.* **2000,** *131*, 889-893.

[19] R. Benassi, C. Bertarini, E. Kleinpeter, F. Taddei, *THEOCHEM.* **2000,** *498*, 217-225.

[20] P. Wilhelm, M. Neuenschwander, *Helv. Chim. Acta* **1999,** *82*, 338-346.

[21] S. Zhu, C. Qin, G. Xu, Q. Chu, *Tetrahedron Lett.* **1998,** *39*, 5265-5268.

[22] M. Dees, H.H. Otto, *Monatsh. Chem.* **1998,** *129*, 689-696.

[23] E. Kleinpeter, A. Koch, M. Heydenreich, S.K. Chatterjee, W.D. Rudorf, *J. Mol. Struct.* **1995,** *356*, 25-33.

[24] S.R. Marder, L.T. Cheng, B.G. Tiemann, A.C. Friedli, M. Blanchard-Desce, J.W. Perry, J. Skindhoej, *Science* **1994**, *263*, 511-514.

[25] R.R. Pappalardo, E.S. Marcos, M.F. Ruiz-Lopez, D. Rinaldi, J.L. Rivail, *J. Am. Chem. Soc.* **1993,** *115*, 3722-3730.

[26] G. Maas, B. Feith, *Synth. Commun.* **1984,** *14*, 1073-1079.

[27] H. Schubert, I. Bast, M. Regitz, *Synthesis* **1983,** *8*, 661-663.

[28] D. Lloyd, H. McNab, *Angew. Chem.* **1976,** *88*, 496-504.

[29] C.R. Prokopp, M.A. Rubin, P.D. Sauzem, A.H. de Souza, D.B. Berlese, R.V. Lourega, M.N. Muniz, H.G. Bonacorso, N. Zanatta, M.A.P. Martins, C.F. Mello, *Braz. J. Med. Biol. Res.* **2006**, *39*, 795–799.

[30] (a) A. Balbi, M. Anzaldi, M. Mazzei, M. Miele, M. Bertolotto, L. Ottonello, F. Dallegri, *Bioorg. Med. Chem*. **2006**, *14*, 5152–5160; (b) M.V. Patel, R. Bell, S. Majest, R. Henry, T. Kolasa, *J. Org. Chem*. **2004**, *69*, 7058–7065; (c) H. Süleyman, M.E. Büyükokuroğlu, *Biol. Pharm. Bull*. **2001**, *24*, 1133-1136.

[31] E. Shchegol'kov, O. Khudina, L. Anikinam, Y. Burgart, V. Saloutin, *Pharm. Chem. J.* **2006**, *40*, 373–376.

[32] D.M. Bailey, P.E. Hansen, A.G. Hlavac, E.R. Baizman, J. Pearl, A.F. Defelice, M.E. Feigenson, *J. Med. Chem*. **1985**, *28*, 256–260.

[33] (a) Z. Dardari, M. Lemrani, A. Sebban, A. Bahloul, M. Hassar, S. Kitane, M. Berrada, M. Boudouma, *Arch. Pharm*., **2006**, *339*, 291–298; (b) R.N. Mahajan, F.H. Havaldar, P.S. Fernandes, *J. Indian Chem. Soc*. **1991**, *68*, 245–246.

[34] W.W. Wardakhan, N.A. Louca, *J. Chil. Chem. Soc*. **2007**, *52*, 1145–1149.

[35] (a) J. Elguero, in *Comprehensive Heterocyclic Chemistry II*, Vol. 3 (Eds: A.R. Katritzky, C.W. Rees, E.F.V. Scriven), Elsevier Ltd **1996**, Ch. *3.01*, pp. 1–75; (b) M. Sutharchanadevi, R. Murugan, in *Comprehensive Heterocyclic Chemistry II*, Vol. 3 (Eds: A.R. Katritzky, C.W. Rees, E.F.V. Scriven), Elsevier Ltd **1996**, Ch. *3.03*, pp 221-260.

[36] (a) D.H. Dawood, E.S. Nossier, M.M. Ali, A.E. Mahmoud, *Bioorg. Chem*. **2020**, *101*, 103916; (b) P. Ravula, H.B. Vamaraju, M. Paturi, J.N.G.N.S. Chandra, *Arch. Pharm. Chem. Life Sci.* **2018**, *351*, e1700234.

[37] (a) J. Sahoo, C.R. Sahoo, P.K. Nandini Sarangi, S.K. Prusty, R.N. Padhy, S.K. Paidesetty, *Eur. Med. Chem.* **2020**, *186*, 111911; (b) Z. Zhao, X. Dai, C. Li, X. Wang, J. Tian, Y. Feng, J. Xie, C. Ma, Z. Nie, P. Fan, M. Qian, X. He, S. Wu, Y. Zhang, X. Zheng, Eur. Med. Chem. **2020**, *186*, 111893; (c) S. Liu, X. Bao, B. Wang, Chem. Commun. **2018**, *54*, 11515; (d) X. Xie, L. Xiang, C. Peng, B. Han, *Chem. Rec.* **2019**, *19*, 2209.

[38] (a) A. Spallarossa, M. Lusardi, C. Caneva, A. Profumo, C. Rosano, M. Ponassi, *Molecules* **2021**, *26*, 557-571; (b) A. Spallarossa, C. Caneva, M. Caviglia, S. Alfei, S. Butini, G. Campiani, S. Gemma, M. Brindisi, D.M. Zisterer, S.A. Bright, C.D. Williams, E. Crespan, G. Maga, G. Sanna, I. Delogu, G. Collu, R. Loddo, *Eur. J. Med. Chem.* **2015**, *102*, 648-660; (c) A. Ranise, F. Lucchesini, M. Caviglia, S. Alfei, A. Spallarossa, C. Caneva, *Tetrahedron* **2013**, *69*, 10858-10868.

[39] W. Ma, B. Peterson, A. Kelson, E. Laborde, *J. Comb. Chem*. **2009**, *11*, 697-703.

[40] M. Verma, V. Gupta, R.K. Nema, U. Misra, *J. Chem. Sci.* **2008**, *6*, 179-184.

[41] G.H. Elgemeie, S.H. Elsayed, A.S. Hassan, *Synt. Comm*. **2008**, *38*, 2700-2706.

[42] N. Rateb, *Phosphorus, Sulfur Silicon Relat. Elem.* **2005**, *180*, 2361-2372.

[43] J.N. Dominguez, J.E. Charris, M. Caparelli, F. Riggione, *Arzneim.-Forsch.* **2002**, *52*, 482-488.

[44] B. Stanovnik, J. Svete, *Sci. Synth.* **2002**, *12*, 15-225.

[45] P. Plath, B. Wuerzer, W. Rohr, *Ger. Offen.* **1979**, DE 2747531 A1 19790426.

[46] F.P.L. Lim, R.X.Y. Gan, A.V. Dolzhenho, *Tetrahedron Lett.* **2017**, *58*, 775-778.

[47] G.M.E. Ali, D.A. Ibrahim, A.M. Elmetwali, N.S. Ismail, *Bioorganic Chemistry* **2019**, *86*, 1-14.

[48] G. Elgemeie, N. Fathy, W. Zaghary, A. Farag, *Nucleosides Nucleotides Nucleic Acids* **2017**, *36*, 198-212.

[49] A.A. Ivashchenko, A.V. Ivashchenko, N.F. Savchuk, *PCT Int. Appl.* **2009**, WO 2009093208 A2 20090730.

[50] Y. Liu, L.B. Tian, H.Y. Yang, H.P. Zhang, *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21*, 3959-3965.

[51] V. De Giorgi, A. Gori, M. Grazzini, S. Rossari, F. Scarfì, S. Corciova, A. Verdelli, T. Lotti, D. Massi, *Expert. Rev. Anticancer Ther.* **2011**, *11*, 739-747.

[52] (a) J. Dobos, *Magy Onkol.* **2009**, *53*, 47-50; (b) J. Dobos, J. Timar, J. Bocsi, Z. Bùrian, K. Nagy, G. Barna, I. Petàk, A. Ladànyi, *Int. J. Cancer* **2004**, *112*, 771-776; (c) B. Richardson, A. Price, M. Wagner, V.

Williams, P. Lorigan, S. Browne, J.G. Miller, S. Mac Neil, *Brit. J. Dermatol.* **1999**, *80*, 2025-2033.

[53] (a) C.G. Thomas, A. Ström, K. Lindberg, J.A. Gustafsson, *Breast Cancer Res. Treat.* **2010**, *127*, 417-427; (b) J.A. Gustafsson, M. Warner, *J. Steroid Biochem. Mol. Biol.* **2000**, *74*, 245-248.

[54] B.E. Fink, D.S. Mortensen, S.R. Stauffer, Z.D. Aron, J.A. Katzenellenbogen, *Chem. Biol.* **1999**, *6*, 205-219.

[55] (a) A. Cheng, N. Garg, L. Krueger, J. Loefstedt, E. Koch, K. Koehler, L. Hagberg, D. Noeteberg, *PCT Int. Appl.* **2013**, WO 2013017654 A1 20130207; (b) D.S. Mortensen, A.L. Rodriguez, K.E. Carlson, J. Sun, B.S. Katzenellenbogen, J.A. Katzenellenbogen, *J. Med. Chem.* **2001**, *44*, 3838-3848.

[56] J. Min, P. Wang, S. Srinivasan, J.C. Nwachukwu, P. Guo, M. Huang, K.E. Carlson, J.A. Katzenellenbogen, K.W. Nettles, H.B. Zhou, *J. Med. Chem.* **2013**, *56*, 3346-3366.

[57] S.R. Stauffer, C.J. Coletta, R. Tedesco, G. Nishiguchi, K. Carlson, J. Sun, B.S. Katzenellenbogen, J.A. Katzenellenbogen, *J. Med. Chem.* **2000**, *43*, 4934-4937.

[58] L.K. Gavrin, A. Lee, B.A. Provencher, W.W. Massefski, S.D. Huhn, G.M. Ciszewski, D.C. Cole, J.C. McKew, *J.Org.Chem.* **2007**, *72*, 1043-1046.

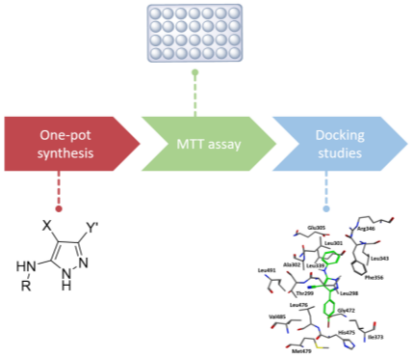
[59] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, A.J. Olson, *J. Comput. Chem.* **2009**, *16*, 2785–2791.

[60] J.C. Nwachukwu, S. Srinivasan, N.E. Bruno, J. Nowak, N.J. Wright, F. Minutolo, E.S. Rangarajan, T. Izard, X.Q. Yao, B.J. Grant, D.J. Kojetin, O. Elemento, J.A. Katzenellenbogen, K.W. Nettles, *Cell Chem. Biol.* **2017**, *24*, 35-45.

[61] L.R Roberts, D. Armor, C. Barker, A. Bent, K. Bess, A. Brown, D.A. Favor, D. Ellis, S.L. Irving, M. Mackenny, C. Phillips, N. Pullen, A. Stennett, L. Strand, M. Styles, *Bioorg. Med. Chem.* **2011**, *21*, 2580-5683.

[62] M.D. Winn, C.C. Ballard, K.D. Cowtan, E.J. Dodson, P. Emsley, P.R. Evans, R.M. Keegan, E.B. Krissinel, A.G.W. Leslie, A. McCoy, S.J. McNicholas, G.N. Murshudov, N.S. Pannu, E.A. Potterton, H.R. Powell, R.J. Read, A. Vagin, K.S. Wilson, *Acta Cryst.* **2011**, *67*, 235-242.

## Entry for the Table of Contents




A one-pot, regioselective and versatile procedure was developed for the synthesis of a series of highly functionalized pyrazole derivatives. The prepared compounds were tested for their antiproliferative activity on a panel of tumour and normal cells. 5-anilino-4-nitrile-3-phenyl pyrazoles emerged as the most promising derivatives and compound **23** selectively inhibited the growth of estrogen-dependent SKMEL-28 and HeLa cells. Docking simulations calculated micromolar Ki values for the ERα-**23** and ERβ-**23** complexes thus suggesting estrogen receptors as potential biological targets for this series of pyrazoles.