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Original article

# Synthesis of *N*-aryl-5-amino-4-cyanopyrazole derivatives as potent xanthine oxidase inhibitors

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**Abstract**

Some pyrazolo[3,4-*d*]pyrimidines, structurally related with allopurinol, a well known xanthine oxidase inhibitor, clinically used in the therapy of gout, have also been reported as potent inhibitors of xanthine oxidase and the growth of several human tumour cell lines. Considering the potential interest of this family of compounds, the aim of the present study was to synthesise and provide a full chemical characterization of new *N*-aryl-5-amino-4-cyanopyrazole derivatives and their corresponding pyrazolo[3,4-*d*]pyrimidines. Their biological activity pertaining to the xanthine oxidase inhibition and effect on the growth of three tumour cell lines (MCF-7, NCI-H460, and SF-268) are also provided. With only one exception, the synthesised compounds showed no effect on the growth of the three tumour cell lines. However, a strong xanthine oxidase inhibitory activity was observed for almost all pyrazolo[3,4-*d*]pyrimidines tested, revealing some of them $IC_{50}$ values below 1 μM. The results of the molecular docking studies of these compounds, against xanthine oxidoreductase are also described, providing an atomistic explanation of the differences in the inhibitory efficiency. MEP calculations were used to explain different inhibitory efficiency of similar inhibitors.


*Keywords:* Nitrogen heterocycles; Aminocyanopyrazole derivatives; Pyrazolo[3,4-*d*]pyrimidines; Xanthine oxidase; Antitumour agents; Cytotoxic; Docking; Computational studies

## 1. Introduction

Pyrazole derivatives constitute an important family of compounds due to their applications as pharmaceuticals, agrochemicals and dyestuffs [1–3]. Several 1-phenylpyrazoles (**1**) have been designed and tested as xanthine oxidase inhibitors [4]. Among them, Y-700 ([1-[3-cyano-4-(2,2-dimethylpropoxy)phenyl]-1*H*-pyrazole-4-carboxylic acid), was shown to be a potent xanthine oxidase inhibitor, and was already studied pertaining to its mechanism of inhibition and pharmacokinetic properties [5]. The results suggested that Y-700 will be a promising candidate for the treatment of hyperuricemia and other diseases in which xanthine oxidase may be involved [5].

The formation of pyrazolo[3,4-*d*]pyrimidines (**2**) from pyrazole derivatives with *ortho*-aminonitriles (**3**) is well documented [6–9]. These compounds are of great pharmacological importance, namely as glycogen synthase kinase-3 (GSK-3) inhibitors [10a] and antihistaminic [10b]. Some pyrazolo[3,4-*d*]pyrimidines, structurally related with allopurinol (**4**), a well known xanthine oxidase inhibitor clinically used in the therapy of gout, have also been reported as potent inhibitors

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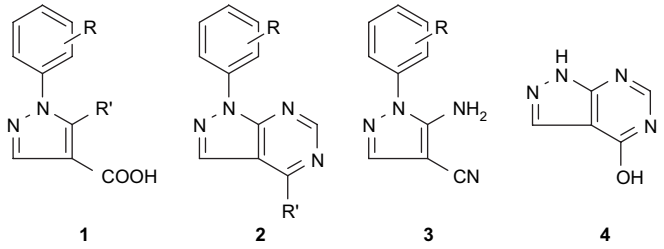
of xanthine oxidase [7,11] and the growth of several human tumour cell lines [6,12–15].




1 2 3 4

Following previous successful work on the synthesis of aryldicyanoaminopyrazoles [16] and arylaminocyanopyrazoles containing groups in the *meta* position of the phenyl ring [17], it was now decided to obtain similar heterocyclic compounds with substitution in the *para* position and also build up a fused pyrimidine ring. The compounds synthesised were tested for their capacity to inhibit xanthine oxidase activity and the *in vitro* growth of three tumour cell lines, MCF-7 (breast cancer), NCI-H460 (non-small cell lung cancer) and SF-268 (CNS cancer).

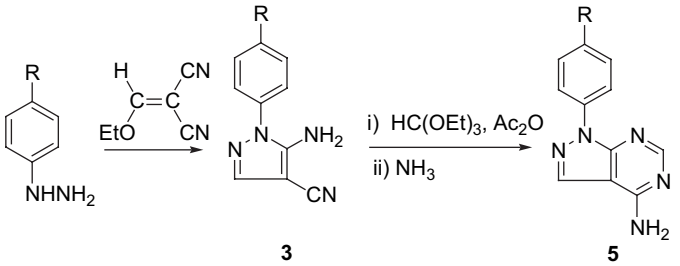
## 2. Results and discussion

### 2.1. Chemistry

In this work a classical method of pyrazole synthesis involving the reaction between hydrazines and β-difunctional compounds was applied for the preparation of compounds (**3**). Aminopyrazoles (**3a**), (**3e**) and (**3g**) are known [6] and the aminopyrazole (**3e**) was used for making azo dyes [18], however, they could not be fully characterized at that time. Recently, in our group, compounds (**3c**) and (**3e**) were prepared, fully characterized and used to make dyes derived from anilines and β-naphthol [19].

Pyrazolopyrimidines (**5**) were prepared (Scheme 1) by a known procedure [20]. Although three of them, (**5a**), (**5e**) and (**5g**), were reported earlier [6], their NMR data are not yet available. The 4-amino group and/or the carboxylic group in these systems confers them great versatility.

Purine derivatives containing aminoacid residues in their molecules have received some attention in cancer chemotherapy [7]. It is also known that a sugar moiety linked to a drug may increase its bioavailability [21]. It was then decided to bind compounds (**5c**) and (**3c**) to glycine or to α-acetobromoglucose.




3 5

R: **a**=Me; **b**=OMe; **c**=$CO_2H$; **d**=CN; **e**=$NO_2$; **f**=$CF_3$; **g**=Cl

Scheme 1. Synthesis of compounds **3** and **5.**

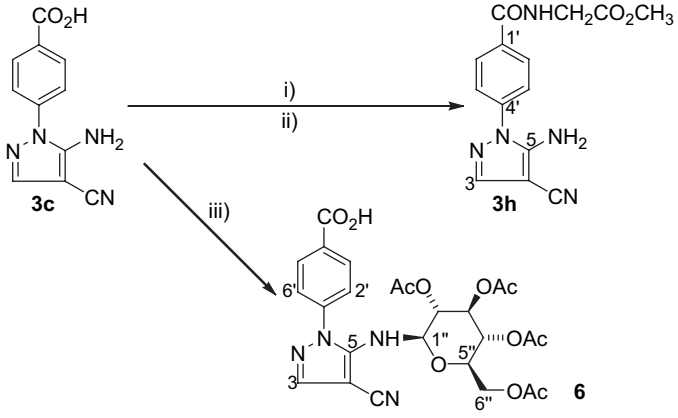
This work started with reaction of the required hydrazines with ethoxymethylenemalononitrile in boiling ethanol. When the arylhydrazine hydrochloride was the starting material the reaction proceeded in the presence of triethylamine, and lower yields were then obtained. The 5-amino-4-cyano-*N*-phenylpyrazoles (**3**) were obtained in yields from 43% (for **3e**) to 89% (for **3c**) (Scheme 1). Aminocyanopyrazoles (**3**) were evidenced on IR by signals at 2215–2243 $cm^{-1}$ and in the NMR spectra by a singlet for proton 3 (e.g. at 7.86 ppm for compound **3d**) and the pair of doublets expected for the *para* substituted phenyl ring. Other techniques such as $^{13}C$, HMQC and HMBC were also used.

Pyrazolo[3,4-*d*]pyrimidines (**5**) were obtained by reflux of the corresponding pyrazoles with triethyl orthoformate in acetic anhydride, to give the imidates which were then, in the crude form, cyclised with ammonia in methanol (Scheme 1). The yields were moderate to good (24% for **5a** and 82% for **5d**) except for (**5e**) where a poor yield was obtained (12%).

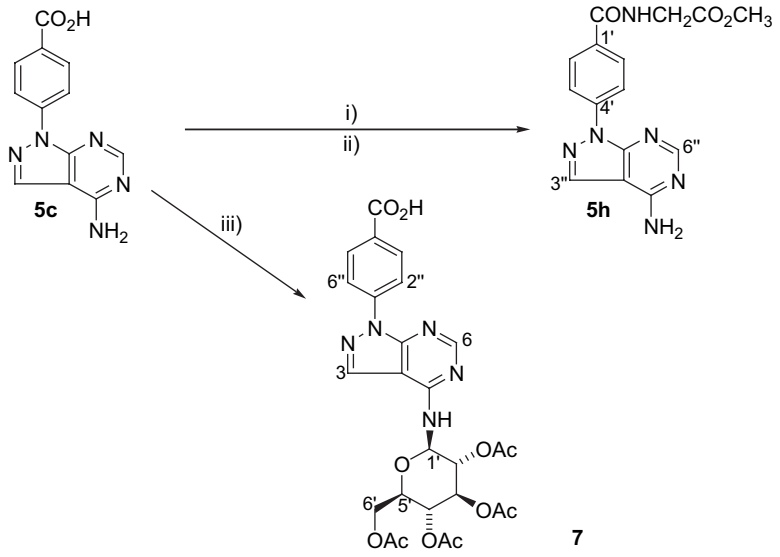
The formation of the pyrazolopyrimidine products was evident from the NMR spectra where two singlets (due to protons 3 and 6) were observed.

The amino group allowed modifications of the molecules through binding to sugars or aminoacids. The alkylations of the pyrazole (**3c**) and pyrazolopyrimidine (**5c**) on the amino group with α-acetobromoglucose in DMSO in the presence of triethylamine were carried out (Schemes 2 and 3). Compounds (**6**) and (**7**) were isolated in 43 and 44% yields, respectively. All the signals due to the glucose ring and the four acetyl groups were observed, and it may also be worth mentioning, e.g. for compound (**7**), two double doublets at 4.33 ($J = 12.6$ and 4.5 Hz) and 4.15 ($J = 12.6$ and 1.8 Hz) for both hydrogen atoms (sugar-$CH_2$OAc) and the ddd at 3.96 ($J = 9.6$, 4.5 and 1.8 Hz) for ring 5′ proton.

Compounds (**3c**) and (**5c**) were also reacted with glycine methyl ester hydrochloride (Schemes 2 and 3), affording the corresponding (**3h**) and (**5h**) in 32 (crude) and 72% yield, respectively, as evidenced by their NMR spectra. For example




Scheme 2. Reaction conditions: (i) HBTU, DMF, RT; (ii) GlyOMe, HCl, TEA, DMF, RT; (iii) DMSO, $NEt_3$, α-acetobromoglucose.




Scheme 3. Reaction conditions: (i) HBTU, DMF, RT; (ii) GlyOMe,HCl, TEA, DMF, RT; (iii) DMSO, $NEt_3$, α-acetobromoglucose.

a typical triplet ($J = 5.7$ Hz) corresponding to NHGly appears at 9.08 and 9.02 ppm for compounds (**3h**) and (**5h**), respectively.

All the compounds were characterized by spectroscopic methods and elemental analysis or high resolution mass spectrometry.

## 2.2. Biological activity

### 2.2.1. Effects of compounds on xanthine oxidase activity

The inhibition of xanthine oxidase activity by aminocyanopyrazoles (**3a**–**3h** and **6**) and pyrazolo[3,4-*d*]pyrimidines (**5a**–**5h** and **7**) was examined at the maximum concentration of 100 μM. Compounds presenting an inhibitory effect higher than 50%, at this concentration, were further tested at a wide range of concentrations in order to determine its $IC_{50}$ values (Table 1). The tested aminocyanopyrazoles showed no appreciable activity except compounds (**3c**) and (**3h**), which exhibited a moderate xanthine oxidase inhibition, with an $IC_{50}$ of $36.11 \pm 1.45$ and $37.92 \pm 3.03$ μM, respectively. However, a strong inhibitory effect was observed for all of the pyrazolo[3,4-*d*]pyrimidines tested. Only compound (**5c**) showed a weak effect ($IC_{50} = 80.97 \pm 4.64$ μM). Among the pyrazolo[3,4-*d*]pyrimidines, the strongest effects were observed in

Table 1
Effect of compounds on xanthine oxidase and on the growth of human tumour cell lines

| Compd | R | XO inhibitory activity $IC_{50}$ (μM)[a] | Inhibition of tumour cell lines GI50 (μM)[b] | | |
|---|---|---|---|---|---|
| **3a** | $CH_3$ | >100 | >150 | >150 | >150 |
| **3b** | $OCH_3$ | >100 | >150 | >150 | >150 |
| **3c** | COOH | $36.11 \pm 1.45$ | ND | ND | ND |
| **3d** | CN | >100 | >150 | >150 | >150 |
| **3e** | $NO_2$ | >100 | $64.01 \pm 4.03$ | $135.02 \pm 9.61$ | $127.51 \pm 7.50$ |
| **3f** | $CF_3$ | >100 | >150 | >150 | >150 |
| **3g** | Cl | >100 | >150 | >150 | >150 |
| **3h** | $CONHCH_2COOCH_3$ | $37.92 \pm 3.03$ | >150 | >150 | >150 |
| **5a** | $CH_3$ | $17.35 \pm 1.19$ | ND | ND | ND |
| **5b** | $OCH_3$ | $19.58 \pm 0.20$ | >150 | >150 | >150 |
| **5c** | COOH | $80.97 \pm 4.64$ | ND | ND | ND |
| **5d** | CN | $0.40 \pm 0.01$ | ND | ND | ND |
| **5e** | $NO_2$ | $2.20 \pm 0.05$ | ND | ND | ND |
| **5f** | $CF_3$ | $0.18 \pm 0.02$ | ND | ND | ND |
| **5h** | $CONHCH_2COOCH_3$ | $0.08 \pm 0.01$ | >150 | >150 | >150 |
| **6** | – | >100 | ND | ND | ND |
| **7** | – | $18.44 \pm 2.59$ | >150 | >150 | >150 |

ND: Not done.

[a] Results are expressed as means ± SEM of 3 independent observations performed in triplicate. Allopurinol was used as positive control (% inhibition (100 μM) = $96.30 \pm 0.30$, $IC_{50} = 24.40 \pm 0.50$ μM).

[b] Results are mean ± SEM of 1–6 independent experiments performed in duplicate. Doxorubicin was used as positive control: $GI_{50}$ (MCF-7) = $42.80 \pm 8.20$ nM; $GI_{50}$ (SF-268) = $93.00 \pm 7.00$ nM; $GI_{50}$ (NCI-H460) = $94.00 \pm 8.70$ nM.

those possessing a glycine methyl ester (**5h**), cyano (**5d**), nitro (**5e**) or trifluoromethyl group (**5f**), exhibiting $IC_{50}$ values near or below 2 μM. Compounds (**5a**), (**5b**), and (**7**) showed an interesting activity with $IC_{50}$ around 20 μM.

Although the number of tested compounds is limited, some structural features, important to the xanthine oxidase inhibitory effect, can be inferred. In general, when comparing the xanthine oxidase inhibitory effect of aminocyanopyrazoles with that of pyrazolo[3,4-*d*]pyrimidines, it can be concluded that the addition of a pyrimidine ring was responsible for an increased inhibition. However, comparing the compounds with a carboxylic group (**3c** and **5c**), that differ only in the presence of a pyrimidine ring, it seems that this ring was responsible for a two-fold decrease of activity. Moreover, the introduction of a sugar linked to the amino group of compound (**5c**) led to a stronger inhibitory effect of compound (**7**). On the contrary, the introduction of the sugar on the amino group of compound **3c** led to a loss of inhibitory effect. The formation of the amide between compound (**5c**) and glycine methyl ester was associated with a strong increase of xanthine oxidase inhibition, but the same structural modification on compound (**3c**) did not affect the inhibitory effect.

In order to evaluate the type of xanthine oxidase inhibition of the most potent aminocyanopyrazoles and pyrazolo[3,4-*d*] pyrimidines studied, their inhibitory effects were tested at different concentrations of the substrate (xanthine), as shown in Fig. 1. Both the $V_{max}$ and $K_m$ values obtained in the presence of the inhibitors are different from the same values obtained in their absence (Table 2), suggesting a mixed non-competitive inhibitory effect, that is the inhibitors bind both to the enzyme and to the xanthine/xanthine oxidase complex, but with greater affinity for the latter [22].

Previous studies on the effects of the introduction of phenyl groups in purines and pyrazolopyrimidines [11] on their activity suggested that there is a hydrophobic bonding region, adjacent to the active site on xanthine oxidase, that can complex aryl groups attached to those systems. The authors found that, in general, the introduction of an aryl group in position 1 of the pyrazole ring or in position 6 (pyrimidine ring) increased the inhibitory activity, particularly when an electron-withdrawing group was present. In our work compounds **5** containing an aryl ring substituted by electron-withdrawing groups ($CF_3$, $NO_2$, CN) showed enhanced activity. These findings are coherent with the observations of the authors above referred [11].

### 2.2.2. Effects of compounds on the growth of human tumour cell lines

The ability of aminocyanopyrazoles (**3a–3h** and **6**) and pyrazolo[3,4-*d*]pyrimidines (**5a–5h** and **7**) to inhibit the *in vitro* growth of MCF-7, NCI-H460 and SF-268 cell lines was also evaluated and the results, given in concentrations that were able to cause 50% of cell growth inhibition ($GI_{50}$), are summarized in Table 1. No activity was observed for the compounds tested even at maximum concentration (150 μM), except for aminocyanopyrazole (**3e**). This compound revealed an inhibitory effect that was two-fold higher for MCF-7 than to the other cell lines. This different cell line response may reflect a possible tumour type-specific sensitivity of this compound for breast cancer cell line.

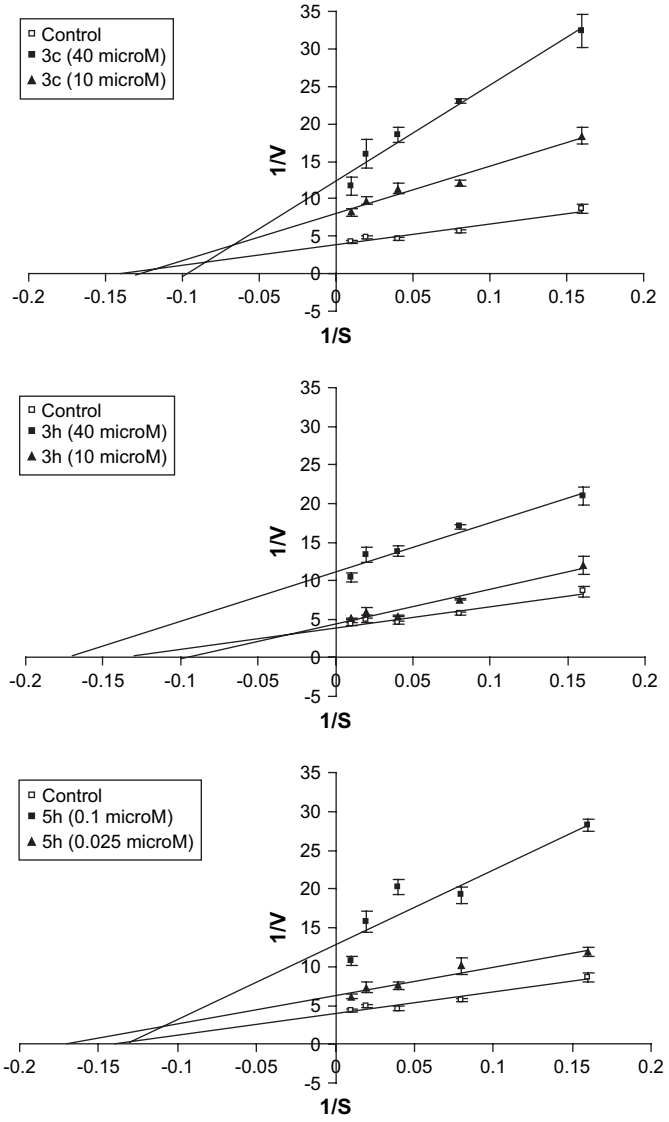



Fig. 1. Lineweaver–Burk plots in the absence (control) and in the presence of inhibitors **3c**, **3h**, and **5h** with xanthine as the substrate. $V = \Delta A/\text{min}$; $S =$ xanthine (μM).

Table 2
$V_{max}$ (enzyme activity) and $K_m$ (apparent substrate activity) for xanthine oxidase in the absence (control) and in the presence of two different concentrations of inhibitors **3c**, **3h**, and **5h**

| | $K_m$ (μM) | $V_{max}$ ($\Delta A$/min) |
|---|---|---|
| Control (xanthine) | 7.16 | 0.26 |
| Xanthine + **3c** (10 μM) | 7.79 | 0.12 |
| Xanthine + **3c** (40 μM) | 10.33 | 0.08 |
| Xanthine + **3h** (10 μM) | 10.41 | 0.23 |
| Xanthine + **3h** (40 μM) | 5.77 | 0.09 |
| Xanthine + **5h** (0.025 μM) | 5.86 | 0.16 |
| Xanthine + **5h** (0.1 μM) | 7.53 | 0.13 |

### 2.3. Computational studies

The known X-ray structures from xanthine oxidoreductase with bound molecules show a highly specific active site. Its overall structure remains almost unchanged from the unbound structure, presenting a narrow tunnel towards the Mo(IV) complex [5,23].

According to our docking studies, all inhibitors bind within four phenylalanine residues, Phe$^{649}$, Phe$^{914}$, Phe$^{1009}$ and Phe$^{1013}$, what is believed to promote the stabilization of the binding positions of the aromatic substrate and might be important for substrate recognition. The presence of these residues makes the region closer to the Mo(IV) complex very tight, which compromises the binding of larger substituted rings. This was observed with all docked inhibitors in which only one phenyl ring was allowed to bind in that region. (See Fig. 2 for compounds **3c**, **5c**, **7**, **5h** and Y-700).

In the active site there are two regions that interact very closely with the docked inhibitors by hydrogen bonds. One region involves Arg$^{880}$, Thr$^{1010}$ and Glu$^{1261}$ (center 1) and the other Asn$^{768}$, Lys$^{771}$ and Glu$^{802}$ (center 2).

Considering the $IC_{50}$ values of all synthesised compounds (Table 1), it is clear that the presence of a pyrazolopyrimidine ring is associated with the substantial increase of the inhibitory effect. The results of docking and molecular electrostatic potential (MEP) studies show that, apart from its size, this molecular moiety can fit inside the active site cavity, interacting very closely with center 2. Moreover, all ligands of this series occupy the same region inside the active site and the substituents at the phenyl ring interact very closely with center 1 pointing towards the Mo(IV) center. The $IC_{50}$ values for compounds **5** show that electron-withdrawing substituents, in general, lead to an increase in the inhibitory effect (**5d**–**5h**). The results point out that in these cases, a tight net of hydrogen bonds with center 1 is generated and a closer interaction with the Mo(IV) center is achieved, thus increasing the protein ligand affinity.

On the other hand compounds of series **3** have higher $IC_{50}$ values, which can be related to the small size and the global linear shape of the structures that preclude an efficient binding with center 1 and center 2 of the active site. Docking results show two possible main binding orientations, and such competitive behaviour may also be responsible for the lower activity for these compounds. One exception is compound **3h** where it appears that a good interaction with both centers of the active site is achieved, improving the ligand binding.

Compounds **6** and **7** have a glucosydic moiety connected to the pyrazolo or pyrazolopyrimidine rings, respectively. For compound **6**, the introduction of a sugar moiety in the 5-amino position of the pyrazole ring resulted in complete loss of activity, as compared with **3c**. For pyrazolopyrimidine **7** the position and the size of the sugar group did not influence its binding inside the active site cavity and closer interactions with center 1 and 2 are still observed. Interestingly, the $IC_{50}$ value for compound **7** is four times lower than for **5c**, although both compounds differ only in the glucosydic group. The result for compound **7** seems to be dependent on extra hydrogen bonds that can be established between the glucosydic moiety and the residues located at the entrance of the binding pocket, namely Asn$^{650}$, Ser$^{744}$ and Lys$^{771}$.

Compounds **3c**, **5c**, **5h** and **7** have structural similarities, however, the $IC_{50}$ values are quite different. The calculated molecular electrostatic potential (MEP) show some tendencies that allow to explain these differences (Fig. 2). These surfaces show areas which are susceptible to electrophilic (red colour) and nucleophilic interactions (blue colour) (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

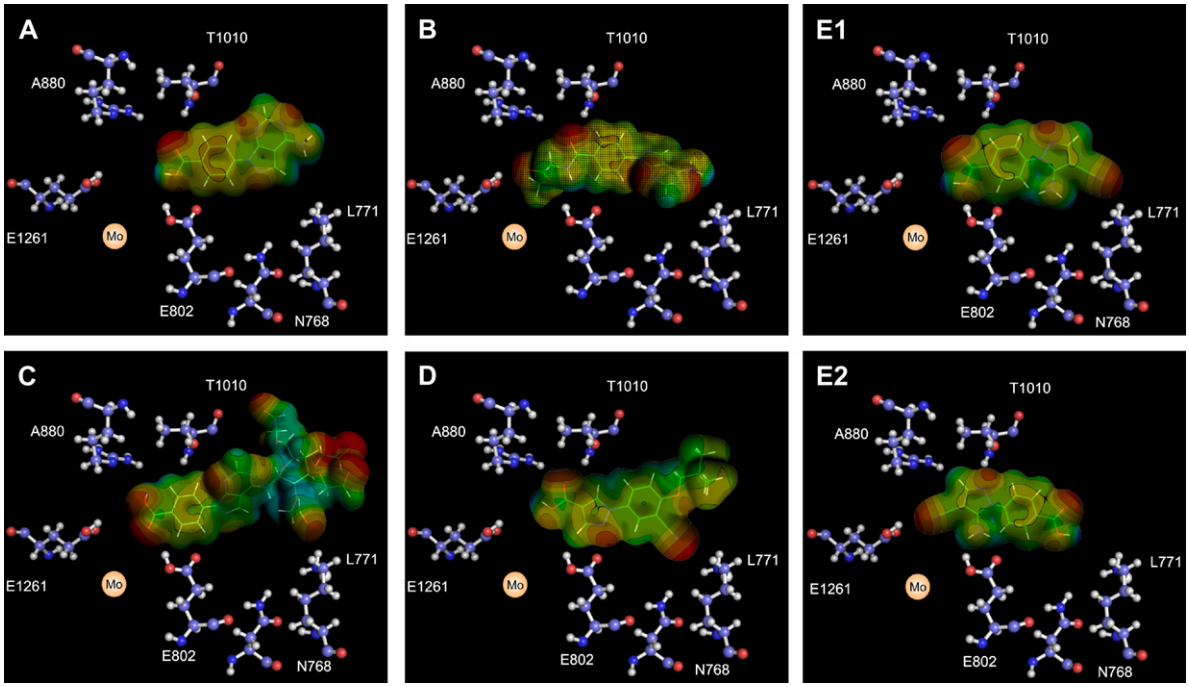



Fig. 2. Docking results and MEP results obtained for compounds **5c** (A), **5h** (B), **7** (C), Y-700 (D) and **3c** (E1, E2).

Compound **5c** has only one region that can establish strong interactions with center 1. Compounds **3c** and **7** show two well-defined regions that are able to interact with center 1 and 2. In addition, compound **7** shows several negative potential regions around the glucosydic moiety that are able to interact with $Asn^{650}$, $Ser^{744}$ and $Lys^{771}$ residues. From these results it can be concluded that these regions are crucial for the inhibitory effect but, their correct alignment with center 1 and 2 is rather important, favouring the formation of strong hydrogen bonds, and therefore enhanced binding.

From all the compounds analysed, pyrazolopyrimidine **5h** has the highest inhibitory effect. The larger substituent at the pyrazolopyrimidine ring did not influence the binding of the ligand and its high flexibility favours a closer interaction with center 1 of the active site and with the Mo(IV) center. The calculated molecular electrostatic potential of this compound shows areas of negative potential similar to those of Y-700, i.e., three well-defined regions susceptible to electrophilic attack, that are specifically directed to the regions of center 1 and 2, where hydrogen bonds occur.

## 3. Conclusions

Sixteen compounds of the pyrazole or pyrazolopyrimidine type (10 of them new) were prepared and fully characterized. Aminopyrazoles showed no significant activity on xanthine oxidase, while pyrazolo[3,4-*d*]pyrimidines showed to be potent inhibitors of this enzyme specially those with cyano (**5d**), nitro (**5e**), trifluoromethyl group (**5f**) or the derivative of glycine methyl ester (**5h**). Concerning the antitumoral activity, the aminocyanopyrazole (**3e**) showed an interesting specificity for the MCF-7 cell line, while the effect of the other tested compounds on the growth of human tumour cell lines was negligible. The results of the molecular docking studies of these compounds against xanthine oxidoreductase provided some explanations of the differences in inhibitory effect. The computational studies concerning ligands of series **3** and **5** lead to the conclusion that the compounds of series **5** showed a better performance for xanthine oxidase inhibition. These results can contribute for the design and development of new xanthine oxidase inhibitors related with the *N*-aryl-5-amino-4-cyanopyrazole scaffold.

## 4. Experimental

### 4.1. Chemistry

#### 4.1.1. General

Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. IR spectra and UV spectra were determined on a Perkin Elmer FTIR-1600 and on a Hitachi U-2000, respectively. $^1$H NMR spectra were recorded at 300 MHz and $^{13}$C NMR spectra were determined at 75.4 MHz both on a Varian Unity Plus Spectrometer. Double resonance, HMQC and HMBC experiments were carried out for complete assignment of proton and carbon signals in the NMR spectra. High resolution mass spectra were obtained on a AutoSpec E spectrometer. Elemental analyses were obtained on a Leco CHNS-932 instrument.

Thin layer chromatography was carried out on pre-coated plates (Merck Kieselgel $60F_{254}$). Column chromatography was performed on silica gel with mixtures of light petroleum and diethyl ether of increasing polarity, unless other conditions are described. Light petroleum refers to the fraction boiling in the range 40–60 °C.

#### 4.1.2. Aminocyanopyrazoles (*3*)

A mixture of arylhydrazine (33 mmol) and ethoxymethylenemalononitrile (33 mmol) was stirred for 30 min and then ethanol (20 mL) was added. The mixture was stirred at room temperature for 24 h. The product, which precipitates, was filtered and recrystallised from ethanol or ethanol–water or other solvent as described. In some cases, as indicated, column chromatography was used. When the arylhydrazine hydrochloride was the starting material, triethylamine, equimolar amount, was added. After stirring the mixture for 5 min, the method described above was followed.

*4.1.2.1. 5-Amino-1-p-tolyl-1H-pyrazole-4-carbonitrile (**3a**).* It was prepared following the general method but the reaction mixture was refluxed for 4 h. A white solid was obtained after recrystallisation from chloroform–diethyl ether (62%, m.p. 149–150 °C; lit. [6] m.p. 158–159 °C). IR (Nujol mull): $\nu = 3333$, 3298, 3181, 2215 (CN), 1661, 1538 cm$^{-1}$. $^1$H NMR (DMSO-$d_6$): $\delta = 2.35$ (s, 3H, $CH_3$), 6.60 (br s, 2H, $NH_2$), 7.31 (d, $J = 8.5$ Hz, 2H, 2′-H and 6′-H), 7.36 (d, $J = 8.5$ Hz, 2H, 3′-H and 5′-H), 7.75 (s, 1H, 3-H). $^{13}$C NMR (DMSO-$d_6$): $\delta = 20.65$ ($CH_3$), 73.23 (C-4), 114.88 (CN), 124.15 (C-3′and C-5′), 129.89 (C-2′ and C-6′), 135.00 (C-1′), 137.47 (C-4′), 141.49 (C-3), 151.15 (C-5). HRMS: calcd. for $C_{11}H_{10}N_4$ $[M]^+$ 198.0905; found 198.0904.

*4.1.2.2. 5-Amino-1-(4′-methoxyphenyl)-1H-pyrazole-4-carbonitrile (**3b**).* A beige solid was obtained after purification by column chromatography (40% ethyl acetate:hexane) (44%, m.p. 128–131 °C). IR (neat): $\nu = 3426$, 3332, 2927, 2217 (CN), 1631, 1563, 1533, 1514 cm$^{-1}$. $^1$H NMR (DMSO-$d_6$): $\delta = 3.79$ (s, 3H, $OCH_3$), 6.54 (br s, 2H, $NH_2$), 7.05 (d, $J = 9.0$ Hz, 2H, 3′-H and 5′-H), 7.37 (d, $J = 9.0$ Hz, 2H, 2′-H and 6′-H), 7.72 (s, 1H, 3-H). $^{13}$C NMR (DMSO-$d_6$): $\delta = 55.48$ ($CH_3$), 72.89 (C-4), 114.57 (C-3′and C-5′), 114.93 (CN), 126.17 (C-2′and C-6′), 130.24 (C-1′), 141.24 (C-3), 151.23 (C-5), 158.80 (C-4′). HRMS: calcd. for $C_{11}H_{10}N_4O$ $[M]^+$ 214.0855; found 214.0857.

*4.1.2.3. 5-Amino-1-(4′-carboxyphenyl)-1H-pyrazole-4-carbonitrile (**3c**) [19].* It was obtained as an orange solid, recrystallised from ethanol–water (89%), m.p. 294–295 °C. $^1$H NMR (DMSO-$d_6$): $\delta = 6.88$ (br s, 2H, $NH_2$), 7.65 (d, $J = 8.7$ Hz, 2H, 2′-H and 6′-H), 7.84 (s, 1H, 3-H), 8.06 (d, $J = 8.7$ Hz, 2H, 3′-H and 5′-H), 13.15 (br s, 1H, COOH).

*4.1.2.4. 5-Amino-1-(4′-cyanophenyl)-1H-pyrazole-4-carbonitrile (**3d**).* It was obtained as a beige solid (78%, m.p.

226–228 °C; no crystallisation was needed). IR (neat): $\nu = 3357$, 3183, 2227 (CN), 1666, 1607, 1538 $cm^{-1}$. $^1H$ NMR (DMSO-$d_6$): $\delta = 6.97$ (br s, 2H, $NH_2$), 7.74 (d, $J = 8.4$ Hz, 2H, 2′-H and 6′-H), 7.86 (s, 1H, 3-H), 7.98 (d, $J = 8.4$ Hz, 2H, 3′-H and 5′-H). $^{13}C$ NMR (DMSO-$d_6$): $\delta = 74.21$ (C-4), 109.90 (C-4′) 114.47 (CN), 118.35 (CN), 124.22 (C-2′and C-6′), 133.72 (C-3′and C-5′), 141.25 (C-1′), 142.81 (C-3), 151.78 (C-5). HRMS: calcd. for $C_{11}H_7N_5$ $[M]^+$ 209.0701; found 209.0703.

*4.1.2.5. 5-Amino-1-(4′-nitrophenyl)-1H-pyrazole-4-carbonitrile (**3e**) [19].* The title compound was obtained as a light yellow solid after recrystallisation from acetone–water (43%), m.p. 222–224 °C (lit. [6] m.p. 224–225 °C). $^1H$ NMR (DMSO-$d_6$): $\delta = 7.06$ (br s, 2H, $NH_2$), 7.83 (d, $J = 9.0$ Hz, 2H, 2′-H and 6′-H), 7.90 (1H, s, 3-H), 8.35 (d, $J = 9.3$ Hz, 2H, 3′-H and 5′-H).

*4.1.2.6. 5-Amino-1-(4′-trifluoromethylphenyl)-1H-pyrazole-4-carbonitrile (**3f**).* It was obtained as a yellowish solid after recrystallisation from ethanol (71%, m.p. 170–171 °C). IR (neat): $\nu = 3457$, 3343, 2214 (CN), 1651, 1614, 1564, 1531 $cm^{-1}$. $^1H$ NMR (DMSO-$d_6$): $\delta = 6.92$ (br s, 2H, $NH_2$), 7.76 (d, $J = 8.5$ Hz, 2H, 2′-H and 6′-H), 7.85 (s, 1H, 3-H), 7.88 (d, $J = 8.5$ Hz, 2H, 3′-H and 5′-H). $^{13}C$ NMR (DMSO-$d_6$): $\delta = 74.00$ (C-4), 114.57 (CN), 122.19 and 125.80 (right side of q, $^1J = 273$ Hz, $CF_3$; it was not possible to assign the other half of this quartet), 124.41 (C-2′and C-6′), 126.72 (C-3′and C-5′), 127.77 (q, $^2J = 32$ Hz, C-4′), 140.89 (C-1′), 142.58 (C-3), 151.74 (C-5). $C_{11}H_7N_4F_3$ (252.06): calcd. C 52.41, H 2.80, N 22.22; found C 52.44, H 2.96, N 22.14. HRMS: calcd. for $C_{11}H_7N_4F_3$ $[M]^+$ 252.0623; found 252.0620.

*4.1.2.7. 5-Amino-1-(4′-chlorophenyl)-1H-pyrazole-4-carbonitrile (**3g**).* It was obtained as a light yellow solid (58%) after recrystallisation from a mixture of chloroform–methanol, m.p. 166–168 °C (lit. [6] m.p. 167–167.5 °C). IR (Nujol mull): $\nu = 3299$, 3175, 2230 (CN), 1665, 1539 $cm^{-1}$. $^1H$ NMR (DMSO-$d_6$): $\delta = 6.76$ (br s, 2H, $NH_2$), 7.52 (d, $J = 8.7$ Hz, 2H, 2′-H and 6′-H), 7.57 (d, $J = 8.7$ Hz, 2H, 3′-H and 5′-H), 7.79 (s, 1H, 3-H). $^{13}C$ NMR (DMSO-$d_6$): $\delta = 73.49$ (C-4), 114.68 (CN), 126.03 (C-2′and C-6′), 129.44 (C-3′and C-5′), 132.24 (C-1′), 136.31 (C-4′), 142.00 (C-3), 151.44 (C-5). $C_{10}H_7N_4Cl$ (218.04): calcd. C 54.87, H 3.22, N 25.60; found C 54.51, H 3.38, N 25.32. HRMS: calcd. for $C_{10}H_7N_4Cl$ $[M+1, {}^{35}Cl]^+$ 219.0437; found 219.0429. $[M+1, {}^{37}Cl]^+$: calcd. 221.0408; found 221.0403.

*4.1.3. Pyrazolopyrimidines (**5a–5g**)*

The required pyrazole (1.0 mmol) was treated with triethyl orthoformate (12.5 mmol) and acetic anhydride (6.0 mmol) and the mixture was refluxed for 24 h. After cooling, the reaction mixture was concentrated to dryness and then complete acetic anhydride removal was ensured by evaporation with 60 mL of $CCl_4$. This residue was stirred with aqueous ammonium hydroxide (25%, 3 × 4 mL, added at different times) for 40–45 min (the pH of the reaction mixture was 12–13) then cooled to 0–5 °C and diluted with distilled water (40 mL). The pH dropped to 10–12. After 30 min stirring at 0–5 °C, the product was filtered and washed with water (10 mL) and dried at 50–60 °C. The product was purified by column chromatography and/or recrystallisation.

*4.1.3.1. 1-p-Tolyl-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (**5a**).* The crude cream coloured solid (86%) obtained was purified by crystallisation (ethanol) followed by column chromatography (ethyl acetate), affording the pure compound (24%), m.p. 223.7–224.3 °C (lit.[6] m.p. >300 °C). IR (neat): $\nu = 3427$ (br), 1660, 1588, 1556, 1514 $cm^{-1}$. $^1H$ NMR (acetone-$d_6$): $\delta = 2.41$ (s, 3H, Me), 7.25 (br s, 2H, $NH_2$), 7.36 (d, $J = 9.3$ Hz, 2H, 2′-H and 6′-H), 8.21 (d, $J = 9.3$ Hz, 2H, 3′-H and 5′-H), 8.33 (1H, s, 3-H), 8.37 (1H, s, 6-H). $^{13}C$ NMR (acetone-$d_6$): $\delta = 20.78$ ($CH_3$), 102.57 (C-3a), 121.46 (C-3′and C-5′), 130.16 (C-2′and C-6′), 133.72 (C-3), 136.37 (C-1′), 138.15 (C-4′), 154.43 (C-4), 157.27 (C-6), 159.42 (C-7a). HRMS: calcd. for $C_{12}H_{11}N_5$ $[M]^+$ 225.1014; found 225.1012.

*4.1.3.2. (4′-Methoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (**5b**).* After recrystallisation (ethanol–water) it was obtained as a light brown solid. Further purification by column chromatography (eluted with ethyl acetate) yielded a light yellow coloured solid (36%), m.p. 218.2–219.8 °C. IR (neat): $\nu = 3418$, 1672, 1658, 1596, 1567, 1522 $cm^{-1}$. $^1H$ NMR (acetone-$d_6$): $\delta = 3.89$ (s, 3H, $OCH_3$), 7.12 (d, $J = 9.3$ Hz, 2H, 3′-H and 5′-H), 7.30 (br s, 2H, $NH_2$), 8.19 (d, $J = 9.3$ Hz, 2H, 2′-H and 6′-H), 8.31 (s, 1H, 3-H), 8.34 (s, 1H, 6-H). $^{13}C$ NMR (acetone-$d_6$): $\delta = 55.78$ ($OCH_3$), 102.39 (C-3a), 114.82 (C-3′and C-5′), 123.15 (C-2′and C-6′), 133.46 (C-3), 133.74 (C-1′), 154.15 (C-4), 157.27 (C-6), 158.80 (C-4′), 159.41 (C-7a). HRMS: calcd. for $C_{12}H_{11}N_5O$ $[M]^+$ 241.0919; found 241.0972.

*4.1.3.3. N-(4′-Carboxyphenyl)-1H-4-aminopyrazolo[3,4-d]pyrimidine (**5c**).* The crude product (54%), was purified by column chromatography (60% methanol–chloroform) followed by recrystallisation (ethanol–water) affording the title compound (25%) as a white solid, which does not melt, but decomposes below 280 °C. IR (neat): $\nu = 3471$–3173 (br), 1666, 1600, 1556 $cm^{-1}$. $^1H$ NMR (DMSO-$d_6$): $\delta = 7.80$ (br s, 2H, $NH_2$), 8.08 (d, $J = 8.7$ Hz, 2H, 2′-H and 6′-H), 8.18 (d, $J = 8.7$ Hz, 2H, 3′-H and 5′-H), 8.30 (s, 1H, 6-H), 8.38 (s, 1H, 3-H), OH not observed. $^{13}C$ NMR (DMSO-$d_6$): $\delta = 101.52$ (C-3a), 119.23 (C-3′and C-5′), 130.14 (C-2′and C-6′), 134.35 (C-3), 135.92 (C-4′), 140.02 (C-1′), 153.42 (C-4), 156.80 (C-6), 158.36 (C-7a), 170.02 (CO). HRMS: calcd for $C_{12}H_9N_5O_2$ $[M]^+$ 225.0756; found 225.0759.

*4.1.3.4. 4-(4′-Aminopyrazolo[3,4-d]pyrimidin-1-yl)-benzonitrile (**5d**).* Obtained as a light brown solid, (82%) which was purified by crystallisation from ethanol–water, m.p. 350.9–351.4 °C. IR (neat): $\nu = 3500$–3286 (br), 2239 (CN), 2218 (CN), 1679, 1602, 1582, 1518 $cm^{-1}$. $^1H$ NMR (DMSO-$d_6$):

$\delta = 7.91$ and 8.08 (2 br s, 2H, $NH_2$), 7.97 (d, $J = 8.7$ Hz, 2H, 2-H and 6-H), 8.33 (s, 1H, 6′-H), 8.41 (s, 1H, 3′-H), 8.48 (d, $J = 9.0$ Hz, 2H, 3-H and 5-H). $^{13}$C NMR (DMSO-$d_6$): $\delta = 101.87$ (C-3′a), 107.72 (C-4), 118.71 (C-3 and C-5), 120.01 (CN), 133.60 (C-2 and C-6), 135.64 (C-3′), 142.47 (C-1), 154.18 (C-4′), 157.09 (C-6′), 158.35 (C-7′a). HRMS: calcd. for $C_{12}H_8N_6$ $[M]^+$ 236.0810; found 236.0811.

*4.1.3.5. 1-(4′-Nitrophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (**5e**).* It was obtained as a grey to light brown solid which was recrystallised twice from acetone–water (12%), m.p. 319–320 °C (lit. [6] m.p. >300 °C). IR (neat): $\nu = 3540$–$3286$ (br), 1681, 1596, 1505 cm$^{-1}$. $^1$H NMR (DMSO-$d_6$): $\delta = 8.00$ and 8.10 (2 br s, 2H, $NH_2$), 8.38 (s, 1H, 6-H), 8.43 (d, $J = 9.6$ Hz, 2H, 2′-H and 6′-H), 8.47 (s, 1H, 3-H), 8.62 (d, $J = 9.3$ Hz, 2H, 3′-H and 5′-H). $^{13}$C NMR (DMSO-$d_6$): $\delta = 101.91$ (C-3a), 119.49 (C-3′and C-5′), 125.21 (C-2′and C-6′), 136.13 (C-3), 144.09 (C-1′), 144.32 (C-4′), 154.46 (C-4), 157.31 (C-6), 158.42 (C-7a). HRMS: calcd. for $C_{11}H_8N_6O_2$ $[M]^+$ 256.0709; found 256.0719.

*4.1.3.6. 1-(4′-Trifluoromethylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (**5f**).* It was purified by recrystallisation (ethanol) and it was obtained as an off-cream coloured solid (34%), m.p. 240.9–242.3 °C. IR (neat): $\nu = 3616$–$3250$ (br), 1660, 1597 cm$^{-1}$. $^1$H NMR (DMSO-$d_6$): $\delta = 7.92$ (d, $J = 9.0$ Hz, 2H, 2′-H and 6′-H), 8.10 (very br s, 2H, $NH_2$), 8.35 (s, 1H, 6-H), 8.43 (s, 1H, 3-H), 8.52 (d, $J = 8.7$ Hz, 2H, 3′-H and 5′-H). $^{13}$C NMR (DMSO-$d_6$): $\delta = 101.71$ (C-3a), 120.15 (C-3′and C-5′), 124.20 (q, $^1J = 270$ Hz, $CF_3$), 125.83(q, $^2J = 32$ Hz, C-4′), 126.49 and 126.54 (C-2′and C-6′), 135.25 (C-3), 142.12 (C-1′), 153.98 (C-4), 157.05 (C-6), 158.37 (C-7a). HRMS: calcd. for $C_{12}H_8N_5F_3$ $[M]^+$ 279.0732; found 279.0737.

*4.1.4. Synthesis of compounds (**3h**) and (**5h**)*

Compound (**3c**) or (**5c**) (6.6 mmol) and HBTU [*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate] (6.6 mmol) were taken in anhydrous DMF (30 mL) and the solution was stirred at RT for 25–30 min. A solution of GlyOMe,HCl in DMF (10 mL), containing triethylamine (TEA, 6.6 mmol), was added and the mixture stirred at room temperature for 24 h. Addition of water (300 mL), and stirring for 2.5 h precipitated the by-products. The aqueous phase was extracted with diethyl ether (100 mL) and ethyl acetate ($2 \times 100$ mL) and the combined organic extracts were dried ($MgSO_4$) and concentrated. Recrystallisation from the appropriate solvent afforded the desired compound.

*4.1.4.1. [4′-(5-Amino-4-cyanopyrazol-1-yl)-benzoylamino]-acetic acid methyl ester (**3h**).* A white solid was obtained after recrystallisation from methanol (32% yield, m.p. 196–198 °C). IR (neat): $\nu = 3370$, 3274, 3138, 2223 (CN), 1751, 1661, 1527, 1507 cm$^{-1}$. $^1$H NMR (DMSO-$d_6$): $\delta = 3.66$ (s, 3H, $OCH_3$), 4.03 (d, $J = 5.7$ Hz, 2H, $CH_2$), 6.84 (s, 2H, $NH_2$), 7.64 (d, $J = 8.7$ Hz, 2H, 2′-H and 6′-H), 7.83 (s, 1H, 3-H), 8.01 (d, $J = 8.4$ Hz, 2H, 3′-H and 5′-H), 9.08 (t, $J = 5.7$ Hz, 1H, NHGly). $^{13}$C NMR (DMSO-$d_6$): $\delta = 41.27$ ($CH_2$), 51.79 ($OCH_3$), 73.76 (C-4), 114.66 (CN), 123.59 (C-2′ and C-6′), 128.57 (C-3′ and C-6′), 132.39 (C-4′), 139.98 (C-1′), 142.26 (C-3), 151.49 (C-5), 165.66 (CO amide), 170.34 (CO ester). $C_{14}H_{13}N_5O_3$ (299.11): calcd. C 56.18, H 4.38, N 23.40; found C 56.03, H 4.44, H 23.62. HRMS: calcd for $C_{14}H_{13}N_5O_2$ $[M]^+$ 299.1018; found 299.1017.

*4.1.4.2. [4′-(4″-Aminopyrazolo[3,4-d]pyrimidin-1″-yl)-benzoylamino]-acetic acid methyl ester (**5h**).* The crude product (72%) was purified by column chromatography (eluent: methanol then by ethanol) followed by recrystallisation from chloroform–methanol 1:1 affording the pure compound as a white solid, m.p. 272–274 °C. IR (Nujol mull): $\nu = 3583$, 3338, 3190, 1740, 1677, 1645, 1600, 1510 cm$^{-1}$. $^1$H NMR (DMSO-$d_6$): $\delta = 3.66$ (s, 3H, $OCH_3$), 4.05 (d, $J = 6.3$ Hz, 2H, $CH_2$), 7.88 (br s, 2H, $NH_2$), 8.04 (d, $J = 8.7$ Hz, 2H, 3′-H and 5′-H), 8.34 (s, 1H, 6″-H), 8.38 (d, $J = 8.7$ Hz, 2H, 2′-H and 6′-H), 8.40 (s, 1H, 3″-H), 9.02 (t, $J = 5.7$ Hz, 1H, NHGly). $^{13}$C NMR (DMSO-$d_6$): $\delta = 41.27$ ($CH_2$), 51.78 ($OCH_3$), 101.64 (C-3″a), 119.56 (C-2′and C-6′), 128.46 (C-3′and C-5′), 130.64 (C-4′), 134.86 (C-3″), 141.41 (C-1′), 153.75 (C-4″), 156.95 (C-6″), 158.33 (C-7″a), 165.94 (CO amide), 170.43 (CO ester). $C_{15}H_{14}N_6O_3$ (326.11): calcd. C 55.21, H 4.32, N 25.76; found C 54.95, H 4.49, N 25.43.

*4.1.5. Synthesis of compounds (**6**) and (**7**)*

An anhydrous DMSO (10 mL) solution of pyrazolopyrimidine (**5c**) or pyrazole (**3c**) (0.39 mmol) and TEA (1.18 mmol) was stirred under a nitrogen atmosphere. After 25 min α-acetobromoglucose (0.39 mmol) was added. The mixture was stirred at RT for 2 h and water (60 mL) was added. A solid precipitated and the suspension was cooled between 0 and 5 °C for 20 min and filtered. This solid was recrystallised from adequate solvent. For the glycosylation of pyrazole the reaction mixture was stirred for 20 h.

*4.1.5.1. N-(2″,3″,4″,6″-Tetra-O-acetyl-β-D-glucopyranosyl)-5-amino-1-(4′-carboxyphenyl)-1H-pyrazole-4-carbonitrile (**6**).* The product was recrystallised from 1:1:1 ethyl acetate:hexane:ethyl ether (12 mL). A pale yellow solid was obtained (43.5% yield), m.p. 190.5–192.3 °C. $[\alpha]_D^{20} = -15.29°$ ($c = 2.55$ in $CHCl_3$). IR (neat): $\nu = 3337$, 2220 (CN), 1748, 1637, 1608, 1531 cm$^{-1}$. $^1$H NMR ($CDCl_3$): $\delta = 2.00$, 2.05, 2.06 and 2.08 (4 s, 12H, $4 \times OCH_3$), 3.96 (ddd, $J = 9.6$, 4.5 and 1.8, 1H, 5″-H), 4.15 (dd, $J = 12.6$ and 1.8 Hz, 1H, 6″-$H_a$), 4.33 (dd, $J = 12.6$ and 4.5 Hz, 1H, 6″-$H_b$), 4.82 (br s, 1H, NH), 5.20 (t, $J = 9.6$ Hz, 1H, 4″-H), 5.37–5.34 (m, 2H, 2″-H and 3″-H), 5.93 (d, $J = 6.9$ Hz, 1H, 1″-H), 7.67 (d, $J = 8.4$ Hz, 2H, 3′-H and 5′-H), 7.68 (s, 1H, 3-H), 8.19 (d, $J = 8.7$ Hz, 2H, 2′-H and 6′-H), OH not observed. $^{13}$C NMR ($CDCl_3$): $\delta = 20.54$ and 20.66 ($4 \times CH_3$), 61.38 (C-6″), 67.79 (C-4″), 70.09 (C-2″ or C-3″), 72.41 (C-2″ or C-3″), 72.84 (C-5′), 92.59 (C-1″), 113.54 (CN), 123.27 (C-3′and C-5′), 128.09 (C-4), 131.93 (C-2′and C-6′), 141.67 (C-1′), 142.00 (C-3), 150.03 (C-5), 163.30 (CO acid), 169.36 (CO acetyl), 169.42 (CO acetyl), 170.03 (CO acetyl), 170.56 (CO

acetyl). HRMS: calcd. for $C_{25}H_{26}N_4O_{11}$ $[M+1]^+$ 559.1676; found 559.1659.

*4.1.5.2. N-(4″-Carboxyphenyl)-N-(2′,3′,4′,6′-tetra-O-acetyl-β-D-glucopyranosyl)pyrazolo[3,4-d] pyrimidine (7).* Recrystallisation from ethanol yielded an off-white solid (43%), m.p. 212–214 °C. $[\alpha]_D^{20} = -6.25°$ ($c = 4.0$ in $CH_2Cl_2$). IR (neat): $\nu = 3430$ (very br), 1746, 1643, 1596 $cm^{-1}$. $^1H$ NMR (acetone-$d_6$): $\delta = 2.02$, 2.03, 2.04 and 2.06 (4 s, 12H, $4 \times OCH_3$), 4.19 (dd, $J = 12.3$ and 2.1 Hz, 1H, 6′-$H_a$), 4.27–4.33 (m, 1H, 5′-H), 4.35 (dd, $J = 12.3$ and 4.5 Hz, 1H, 6′-$H_b$), 5.22 (app t, $J = 9.9$ and 9.3 Hz, 1H, 4′-H), 5.33 (dd, $J = 9.9$ and 8.4 Hz, 1H, 2′-H), 5.55 (t, $J = 9.6$ Hz, 1H, 3′-H), 6.19 (d, $J = 8.4$ Hz, 1H, 1′-H), 7.46 (br s, 1H, NH), 8.21 (d, $J = 9.0$ Hz, 2H, 2″-H and 6″-H), 8.427 (s, 1H, 3-H), 8.430 (s, 1H, 6-H), 8.67 (d, $J = 9.0$ Hz, 2H, 3″-H and 5″-H), OH not observed. $^{13}C$ NMR (acetone-$d_6$): $\delta = 29.29$, 29.54, 30.06 and 30.32 ($4 \times CH_3$), 62.42 (C-6′), 68.97 (C-4′), 71.21 (C-2′), 73.00 (C-3′), 73.31 (C-5′), 93.15 (C-1′), 102.98 (C-3a), 120.60 (C-2″and C-6″), 126.30 (C-4″), 131.79 (C-3″and C-5″), 135.46 (C-3), 144.91 (C-1″), 155.56 (C-4), 157.83 (C-6), 159.43 (C-7a), 164.39 (CO), 169.99 (CO), 170.02 (CO), 170.25 (CO), 170.68 (CO). HRMS: calcd. for $C_{26}H_{27}O_{11}N_5$ $[M]^+$ 586.1785; found 586.1786.

### 4.2. Biological assays

#### 4.2.1. Xanthine oxidase assay

The reaction mixture containing 50 mM potassium dihydrogen phosphate buffer, pH 7.4, xanthine oxidase (0.066 U/mL), and a solution of test compounds in DMSO was incubated at room temperature, during 15 min. The reaction was started by addition of xanthine (100 μM) in the presence of EDTA (1 mM), and uric acid formation was followed by measure of absorbance at 295 nm during 2 min. Each study corresponds to three experiments, performed in triplicate.

Final concentrations of DMSO (1%) did not interfere with enzyme activity. Allopurinol was used as positive control.

Additionally, this procedure was repeated for the most potent aminocyanopyrazoles and pyrazolo[3,4-*d*]pyrimidines, tested with several concentrations of xanthine (100, 75, 50, and 25 μM), in order to evaluate the type of inhibition using the Lineweaver–Burk plot.

#### 4.2.2. Tumour cell growth assay

The effects of compounds on the growth of tumour cell lines MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and SF-268 (CNS cancer) were evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) for the *in vitro* anticancer drug screening that use the protein-binding dye sulforhodamine B (SRB) to assess growth inhibition [24,25]. Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine and 50 μg/mL of gentamicin at 37 °C in a humidified atmosphere containing 5% $CO_2$. The optimal plating density of each cell line, that ensure exponential growth throughout all the experimental period, was the same as originally published [24] and was, respectively, $7.5 \times 10^4$ cells/mL to NCI-H460, $1.5 \times 10^5$ cells/mL to MCF-7 and SF-268. Cells were exposed for 48 h to five concentrations of compounds starting from a maximum concentration of 150 μM. Compounds, prepared in DMSO, were freshly diluted with cell culture medium just prior the assays. Final concentrations of DMSO (≤0.25%) did not interfere with the cell lines growth. For each test compound and for each cell line a dose-response curve was generated and the growth inhibition of 50% ($GI_{50}$), corresponding to the concentration of compound that inhibits 50% of the net cell growth was determined as described [24].

### 4.3. Modelling and computational details

The crystal structure of xanthine oxidoreductase was retrieved from the PDB database with the corresponding entry code 1VDV (resolution 1.98 Å) [5]. The protein was prepared by removing all water molecules and adding all hydrogen atoms using the Insight software [26]. The ligands, once built in Insight, were docked onto the active site using the molecular docking software GOLD [27,28].

GOLD is a program for calculating the docking modes of small molecules into protein-binding sites. A genetic algorithm is used to search the best binding of a ligand inside the active site cavity and two different scoring functions can then be used for analysing the obtained results. In this study we have used ChemScore [29], a scoring function that is derived from regression against ligand–receptor binding free energies. The validation of this score function was performed with two X-ray structures of the enzyme xanthine oxidoreductase with bounded inhibitors: 1VDV (inhibitor Y-700) [5] and 1N5X (inhibitor: TEI-6720) [23]. In the docking process the active site was defined as a group of residues that are located within a radius of 15 Å from the carbon atom 15 243 of the phenyl group of the residue $Phe^{1009}$. For each ligand 10 docking runs were performed.

Molecular electrostatic potentials (MEPs) of the ligands were generated at the B3LYP/6-31G* level using Gaussian [30] . We used MOLEKEL [31] to map the electrostatic potential onto an electron density surface of 0.002 electrons/$bohr^3$ (generally used, corresponding to about 95% of the electronic charge) and to draw three-dimensional electrostatic potential isosurfaces. The latter are used to predict long-range interactions.

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