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RESEARCH ARTICLE

Synthesis of aminocyanopyrazoles via a multi-component reaction and anti-carbonic anhydrase inhibitory activity of their sulfamide derivatives against cytosolic and transmembrane isoforms

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Abstract
A convenient protocol for the multicomponent reaction (MCRs) between malononitrile with an orthoester and hydrazine derivatives, under acid catalyst is described. A series of aminocyanopyrazoles **4** was prepared, isolated and characterized. These pyrazoles reacted with sodium nitrite followed by secondary amine reagent and with formic acid to lead pyrazolotriazines **6** and pyrazolopyrimidinones **7**. Some of the aminopyrazoles were converted to the corresponding sulfamides by reaction with sulfamoyl chloride. The aminopyrazoles incorporating phenyl and tosyl moieties were tested as inhibitors of four carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the human (h) hCA I, II, IX and XII. Many of them showed low micromolar or submicromolar inhibition of these enzymes. The corresponding sulfamides were low nanomolar CA inhibitors.

Keywords: MCR aminocyanopyrazoles, pyrazolo[3,4-d][1,2,3]triazines, pyrazolo[4,3-d]pyrimidin-7-ones, carbonic anhydrase inhibitor

Introduction

Due to the convenience and high degree of atom economy, multicomponent reactions (MCRs) have become one of the most efficient tools for rapid scaffold construction and introduction of molecular diversity[1,2]. MCRs are important synthetic tools that can be useful in the preparation of biologically active compounds[3]. In our search for operationally simple, resource and cost-effective processes, we have been investigating pyrazole MCRs. Their classical syntheses in two steps were well described in the literature[4–6]. In fact, pyrazoles constitute an important family of compounds[7,8] due to their applications as antiviral[9], as xanthine oxidase inhibitors[10], as dual MAO-B inhibitors and anti-inflammatory analgesics[11] and have considerable chemical and pharmacological importance[12–15]. In this work, our objective is to achieve valuable MCR aminocyanopyrazoles and to study their application by diazotation in hydrochloric acid and by action of formic acid.

Results and discussion

Synthesis of 5-amino-4-cyano-1-substituted pyrazoles 4

The first step of our strategy was the formation of MCR 5-amino-4-cyano-1- substituted pyrazoles **4**. Several works mention the synthesis of the 5-amino-4-cyano pyrazoles[4–6] prepared via a standard addition of hydrazine derivatives to ketene ethoxymethylene compounds. However, we were delighted to observe the sole formation of pyrazoles **4** when introducing equivalent amounts of malononitrile **1** with orthoester **2** and hydrazine
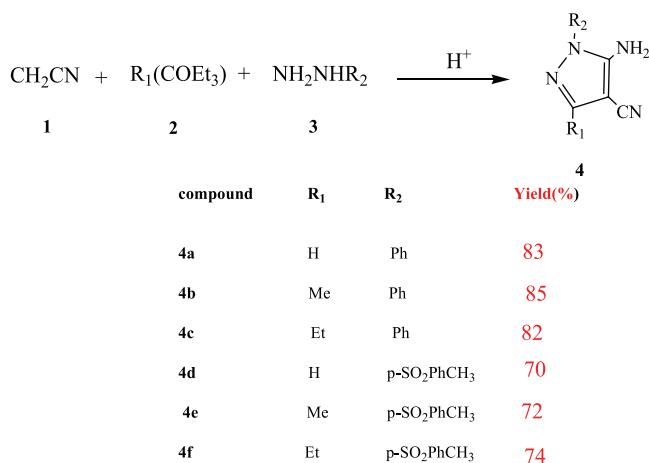
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derivatives **3** in the presence of few drops of acetic acid in the ethanol heated under reflux overnight. This procedure gave satisfying results: phenyl hydrazines afforded the pyrazole in good yields (Scheme 1).

Synthesis of pyrazolotriazines 6 and pyrazolopyrimidinones 7

Taking advantage of the presence of the two functional groups in 5-amino-4-cyano-1-substituted pyrazoles **4**, we prepared the 4-chlorotriazine by diazotation in hydrochloric acid[16–20]. In the literature amino cyano pyrazoles undergo diazotization reaction with $NaNO_2$ in a mixture of HCl/AcOH and leads to the pyrazolotriazin-4-ones[21]. To the best of our knowledge 5-substituted chloropyrazolotriazines **5a–c** are not described in the literature. The chlorine atom was easily substituted by a secondary amine to afford compounds **6a–c**. We note that the mass spectra of triazines **5** and **6** showed a molecular mass decrease by the mass of two nitrogen atoms. This is has been mentioned in literature[22].

The reaction of formic acid in the presence of H_2SO_4 on anthranilonitrile and analogs is well described in the literature[23–27]. We used classical conditions to synthesize compound **7**. 5-amino-4-cyano-1-substituted pyrazoles **4** was refluxed with formic acid in the presence of H_2SO_4 under stirring providing pyrazolopyrimidinones **7** from good to very good yields (Scheme 2). This protocol allows to generalize the synthesis of pyrazolo pyrimidinones and increase in some cases the yield of the reaction. The structures of compounds **7** were determined by IR, ^{1}H, ^{13}C NMR spectra, mass spectroscopy and elemental analysis. These data spectrum demonstrate in particularly the disparities of CN group in the aminocyanopyrazoles.

Some of the amino-containing pyrazoles investigated here were converted to the corresponding sulfamides by reaction with sulfamoyl chloride. It is in fact well know that the sulfamide function is a good zinc-binding group for generating potent CA inhibitors[28,29]. Several such compounds, of type 8a-8c were prepared in this way.

Scheme 1.

compound	R_1	R_2	Yield(%)
4a	H	Ph	83
4b	Me	Ph	85
4c	Et	Ph	82
4d	H	p-SO_2PhCH_3	70
4e	Me	p-SO_2PhCH_3	72
4f	Et	p-SO_2PhCH_3	74

Carbonic anhydrase inhibitory action

Compounds **4a–f** and **8a–c** have been tested as inhibitors of four carbonic anhydrase (CA, EC 4.2.1.1) isoforms, the human (h) hCA I, II, IX and XII (Table 1). The inhibition/activation of CAs are well understood processes, with most classes of inhibitors binding to the metal center[28–34], whereas activators bind at the entrance of the active site cavity and participate in proton shuttling processes between the metal ion – bound water molecule and the environment[35]. It should be mentioned that recently other inhibition mechanisms than the binding to the metal center were reported for α-CAs, which do not directly involve the metal ion from the enzyme active site. For example polyamines bind to the enzyme by anchoring to the zinc-coordinated water/hydroxide ion[36], whereas coumarins act as prodrugs and bind at the entrance of the active site cavity, rather far away from the metal ion[37–39]. The compounds **4** reported here contain an amino moiety attached to the pyrazole ring which may bind to the enzyme similar to the polyamine spermine, which is anchored by means of one of its primary amino moieties to the zinc-coordinated water molecule[36]. Furthermore, some secondary/tertiary sulfonamides as those present

compound	R_1	Yield(%)
5a	H	79
5b	Me	75
5c	Et	76
6a	H	79
6b	Me	75
6c	Et	76
7a	H	82
7b	Me	86
7c	Et	90
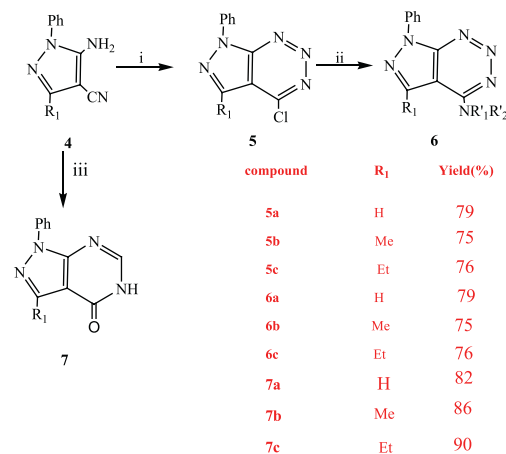
i:$NaNO_2$, HCl 37%, 0-5 °C, ii:-NR'$_1$NR'$_2$(—N◯O), reflux 4h, iii: HCOOH, H_2SO_4

Scheme 2.

Table 1. hCA I, II, IX and XII I nhibition data with compounds **1a–1c** and **4**.

Compound	Ki (μM)*			
	hCA I	hCA II	hCA IX	hCA XII
4a	12.6	6.21	0.34	0.49
4b	10.1	7.14	0.29	0.36
4c	9.4	5.37	0.27	0.44
4d	5.4	0.79	1.24	3.12
4e	5.9	1.13	0.75	0.49
4f	3.1	0.82	0.47	0.15
1a	0.12	0.064	0.022	0.008
1b	0.09	0.051	0.011	0.012
1c	0.08	0.024	0.010	0.010

[a]Mean from 3 different assays by a CO_2 hydrase, stopped flow assay[41].

in derivatives **4d–f**, were recently shown to be inhibitor, probably binding at the entrance of the cavity, in the coumarin-binding site[40].

Data of Table 1 show that compounds **4** act as medium potency, micromolar or submicromolar inhibitors of the four investigated CA isoforms. Against the cytosolic hCA I they showed activity with inhibition constants in the range of 3.1–12.6 µM[41–45]. Sulfonamides **4d–f** were more active than the corresponding phenyl derivatives **4a–c**. The second cytosolic isoform, hCA II, the physiologically dominant one, was better inhibited by compounds **4** compared to hCA I, with K_Is in the range of 0.79–7.14 µM. The structure activity relationship (SAR) was also similar for the two isoforms.

The tumor-associated, transmembrane isoforms hCA IX and XII were better inhibited by compounds **4** compared to the cytosolic ones mentioned above. Thus, against hCA IX, compounds **4** showed inhibition in the range of 0.27–1.24 µM, with the phenyl derivatives more active than the sulfonamides in this case. For hCA XII the inhibition constants were in the range of 0.15–3.12 µM and the SAR more complicated as the best inhibitor was the sulfonamide **4f**, but the phenyl derivatives **4a–c** also showed quite significant inhibition (Table 1).

As these compounds may not bind to the Zn(II) ion from the CA active site, we hypothesize that they inhibit the enzyme either binding similar to spermine (anchoring to the zinc-coordinated water molecule) or as the coumarins, at the entrance of the active site cavity. This hypothesis should be verified by solving the X-ray crystal structure of adducts of these compounds with some CA isoforms[46].

The sulfamides **8** were much more potent inhibitors of all CA isoforms compared to the previously mentioned compounds, being low nanomolar inhibitors of all four CA isoforms investigated here (Table 1). This is probably due to their direct binding to the metal ion within the enzyme active site[28].

In conclusion, we report a novel multicomponent reaction of malononitrile with orthoesters and hydrazine derivatives that leads to the aminocyanopyrazoles **4** with good yields. These pyrazoles are open for further transformation due to an already existing amino and cyano groups in their structures. These pyrazoles react with sodium nitrite followed by secondary amine reagent and with formic acid to lead respectively to pyrazolotriazines **6** and pyrazolopyrimidinones **7**. This new protocol was employed for the rapid synthesis of compounds **5** and **6** and for the amelioration of yield of compounds **7.** Many of therse compounds showed interesting CA inhibitory activity.

Materials and methods

Anhydrous solvents and all reagents were purchased from Sigma-Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and

syringes techniques to transfer solutions. IR spectra were determined in KBr on a JASCO FT-IR-420 spectrometer which precision is of $2\,cm^{-1}$ covering field 400–$4000\,cm^{-1}$. The spectra of NMR ^{1}H and NMR ^{13}C were recorded on an AC Bruker 250 MHz or Bruker Advance III 400 MHz spectrometers in $CDCl_3$ or DMSO-d_6 and the chemical shifts are expressed in ppm. The multiplicities of the signals are indicated by the following abbreviations: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet, brs: broad singlet and the coupling constants are expressed in Hz. The assignment of exchangeable protons (O*H* and N*H*) was confirmed by the addition of D_2O. The melting points were determined in Electrothermal 9100 apparatus and are uncorrected. The reactions were monitored by thin layer chromatography (TLC) using aluminium sheets with silica gel 60 F_{254} (230–400 mesh ASTM) as the stationary phase and ethylacetate/*n*-hexane or MeOH/DCM were used as eluants. The mass spectrometer was operated in EI mode at 70 eV and MS spectra were recorded from *m/z* 50 to 650.

General procedure for synthesis of aminocyanopyrazoles (4)

A solution of malononitrile **1** (33 mmol), orthoester **2** (34 mmol), hydrazine (33 mmol) **3** and few drops of acetic acid in ethanol (30 mL) was heated under reflux overnight. The product, which precipitates, was filtered and recrystallized from ethanol.

5-Amino-1-phenyl-1H-*pyrazolo-4-carbonitrile (4a)*

Yield 83%, white solid; mp = 166°C (methanol); IR (cm⁻¹): 1594, 1640, 2217 (CN), 2917, 3432, 3221, ^{1}H NMR (δ ppm, DMSO): 6.82 (2H, s, NH_2), 7.40–7.55 (6H, m), ^{13}C NMR (δ ppm, DMSO): 144.60 (C-3), 73.48 (C-4), 152.74 (C-5), 114.17 (CN), 117.08–137.52 (C-arom).

5-Amino-3-methyl-1-phenyl-1H-*pyrazolo-4-carbonitrile (4b)*

Yield 85%, white solid; mp = 133°C (methanol); IR (cm⁻¹) 1598, 1645, 2215 (CN), 3331, 3227, ^{1}H NMR (δ ppm, DMSO) 2.15 (3H, s, CH_3), 6.66 (2H, s, NH_2), 7.35–7.52 (5H, m);^{13}C NMR (δ ppm, DMSO) 11.28 (CH_3), 74.23 (C-4), 115.57 (CN), 124.40–138.03 (C-arom), 150.56 (C-5), 151.96 (C-3); MS m = e/z 199 [M + 1]⁺, 100%.

5-Amino-3-ethyl-1-phenyl-1H-*pyrazolo-4-carbonitrile (4c)*

Yield 82%, white solid; mp = 137°C (methanol); IR (cm⁻¹) 1597, 1647, 2209 (CN), 3433, 3296; ^{1}H NMR (δ ppm, DMSO) 1.18 (3H, t, *J* = 7.1 Hz, CH_3), 2.53 (2H, q, *J* = 7.1 Hz, CH_2), 6.61 (2H, s, NH_2), 7.35–7.53 (5H, m); ^{13}C NMR (δ ppm, DMSO) 12.88 (CH_3), 21.13 (CH_2), 73.22 (C-4), 115.47 (CN), 124.43–138.11 (C-arom), 152.13 (C-3), 155.58 (C-5); MS: m = e/z 213 [M + 1]⁺, 100%, 158 [M-54]⁺, 5%.

5-Amino-1-tosyl-1H-*pyrazolo-4-carbonitrile (4d)*

Yield 70%, white solid; mp = 192°C (methanol); IR (cm⁻¹) 1527, 1616, 2211 (CN), 3347, 3225; ^{1}H NMR (δ ppm, DMSO) 2.27 (3H, s, CH_3), 7.11 (2H, s, NH_2), 7.13–7.51 (5H, m); ^{13}C NMR (δ ppm, DMSO) 21.28 (CH_3), 75.51

(C-4), 113.81 (CN), 138.58 (C-3), 145.55 (C-5), 124.66–129.07 (C-arom).

5-Amino-3-methyl-1-tosyl-1H-pyrazolo-4-carbonitrile (4e)

Yield 72%, white solid; mp = 185°C (methanol); IR (cm^{-1}) 1534, 1628, 2216 (CN), 3381, 3227; ^{1}H NMR (δ ppm, DMSO) 2.04 (3H, t, CH$_3$), 2.39 (3H, s CH$_3$), 7.63 (2H, s, NH$_2$), 7.47–7.87 (4H, m); ^{13}C NMR (δ ppm, DMSO) 13.06 (CH$_3$), 21.53 (CH$_3$), 73.97 (C-4), 113.71 (CN), 127.89–133.44 (C-arom), 146.80 (C-3), 154.87 (C-5).

5-Amino-3-ethyl-1-tosyl-1H-pyrazolo-4-carbonitrile (4f)

Yield 74%, white solid; mp = 178°C (methanol); IR (cm^{-1}) 1526, 1643, 2215 (CN), 3346, 3222; ^{1}H NMR (δ ppm, DMSO) 0.87 (3H, t, J = 7.0 Hz, CH$_3$), 2.16 (2H, q, J = 7.0 Hz, CH$_2$), 2.38 (3H, s, CH$_3$), 7.38 (2H, s, NH$_2$) 7.38–7.68 (5H, m); ^{13}C NMR (δ ppm, DMSO) 10.81 (CH$_3$), 21.52 (CH$_2$), 23.30 (CH$_3$), 73.50 (C-4), 114.32 (CN), 128.20–136.42 (C-arom), 143.49 (C-3), 144.26 (C-5).

General procedure for synthesis of chloropyrazolotriazines (5)

A solution of sodium nitrite (11.4 mmol) in water (7 mL) was added over 15 min to a suspension of the foregoing 5-amino-4-cyano-1-substituted pyrazole (8.1 mmol) at 0-5°C in concentrated hydrochloric acid (16 mL). The resulting mixture was stirred at 0°C for a further 40 min and then allowed to stand at room temperature overnight. The reaction mixture was quenched in water (100 mL). The precipitate was washed twice with 15 mL of water, and dried under room temperature, recrystallized in methanol.

4-Chloro-7-phenyl-7H-pyrazolo[3,4-d][1,2,3]triazine(5a)

Yield 79%; mp = 170°C [methanol]; IR (cm^{-1}) 1590 (C=N); NMR ^{1}H (δ ppm, DMSO) 7.75 (1H, s, H$_5$), 7.23 ppm (5H, s); ^{13}C NMR (δ ppm, DMSO) 93.01 (C-4a), 131.15 (C-7a), 136.63 (C-5), 159.87 (C-4), 124.54–129.33 (C-arom); GC-MS: m/z (%) 204 (100), 154 (24), 141 (17); C$_{10}$H$_6$ClN$_5$ Calculated (%): C 51.85, H 2.61, N 30.23, Found (%) C 51.79, H 2.60, N 30.25.

4-Chloro-5-methyl-7-phenyl-7H-pyrazolo[3,4-d][1,2,3]triazine(5b)

Yield 75% mp = 159°C [methanol]; IR (cm^{-1}) 1586 (C=N); ^{1}H NMR (δ ppm, DMSO) 2.38 (3H, s, CH$_3$), 7.26–7.64 ppm (5H, m); ^{13}C NMR (δ ppm, DMSO) 13.52 (CH$_3$), 94.54 (C-4a), 112.26 (C-7a), 137.38 (C-5), 153.08 (C-4), 120.01-133.11 (C-arom); GC-MS: m/z (%)218 (100), 168 (36), 155 (14); C$_{11}$H$_8$ClN$_5$ Calculated (%): C 53.78, H 3.28, N 28.51, Found (%) C 53.79, H 3.30, N 28.53.

4-Chloro-5-ethyl-7-phenyl-7H-pyrazolo[3,4-d][1,2,3]triazine(5c)

Yield 76%; mp = 179°C [methanol]; IR (cm^{-1}) 1587 (C=N); NMR ^{1}H (δ ppm, DMSO) 1.35 (3H, t, J = 7.1 Hz, CH$_3$), 2.80 (2H, q, J = 7.1 Hz, CH$_2$), 7.51 ppm (5H, s); ^{13}C NMR (δ ppm, DMSO) 12.74 (CH$_3$), 21.52 (CH$_2$), 93.13 (C-4a),

112.01 (C-7a), 137.03 (C-5) 157.91 (C-4), 125.04-132.89 (C-arom), GC-MS: m/z (%) = 232 (100), 204 (43), 182 (51), 169 (15), 142 (30), C$_{12}$H$_{10}$ClN$_5$ Calculated (%): C 55.50, H 3.88, N 26.97, Found (%) C 55.48, H 3.89, N 26.96.

General procedure for synthesis of aminopyrazolotriazines (6)

The chloropyrazolotriazine 5a–c (1 mmol) and corresponding amine (10 mL) were refluxed for 6 h. The mixture was cooled at room temperature. When a precipitate was formed, it was filtered, washed twice with 15 mL of water and twice with 8 mL of diethyl ether, and dried at room temperature overnight. The products were recrystallised in methanol to give products 6.

4-Morpholino-7-phenyl-7H-pyrazolo[3,4-d][1,2,3]triazine (6a)

Yield 79% mp = 170°C [methanol]; IR (cm^{-1}) 1556 (C=N), 1453 (C=N), 1422 (N=N); NMR ^{1}H (δ ppm, DMSO) 3.15 (4H, t, J = 7.1 Hz, CH$_2$), 3.64 (4H, t, J = 7.1 Hz, CH$_2$), 7.34-7.68 ppm (5H, m), 7.75 (1H, s, H$_5$); ^{13}C NMR (δ ppm, DMSO) 51.10 (CH$_2$), 68.12 (CH$_2$), 115.15 (C-7a), 93.01 (C-4a), 156.63 (C-5), 159.87 (C-4), 124.54 (C-arom); GC-MS m/z (%) 255 (100), 197 (23), 119 (66), C$_{14}$H$_{14}$N$_6$O Calculated (%): C 59.50, H 5.00, N 29.77, O 5.67 Found (%) C 59.52, H 5.03, N 29.78, O 5.65.

5-Methyl-4-morpholino-7-phenyl-7H-pyrazolo[3,4-d][1,2,3]triazine (6b)

Yield: 75%. mp = 159°C [methanol]. IR (cm^{-1}): 1560 (C=N), 1467 (C=N), 1431 (N=N); NMR ^{1}H (δ ppm, DMSO): 2.28 (3H, s, CH$_3$), 3.17 (4H, t, J = 7.1 Hz, CH$_2$), 3.66 (4H, t, J = 7.1 Hz, CH$_2$); 7.35-7.62 ppm (5H, m); ^{13}C NMR (δ ppm, DMSO) 13.31 (CH$_3$), 50.53 (CH$_2$), 66.63 (CH$_2$), 82.18 (C-4a), 115.39 (C-7a), 152.41 (C-5), 153.80 (C-4), 124.17–139.23 (C-arom); GC-MS m/z (%) 269 (100), 211 (53), 133 (22), C$_{15}$H$_{16}$N$_6$O: Calculated(%): C 60.80, H 5.44, N 28.36, O 5.40 Found (%): C 60.82, H 5.45, N 28.34, O 5.42.

5-Ethyl-4-morpholino-7-phenyl-7H-pyrazolo[3,4-d][1,2,3]triazine (6c)

Yield 76%; mp = 170°C [methanol]; IR (cm^{-1}) 1590 (C=N), 1526 (C=N), 1460 (N=N); NMR ^{1}H (δ ppm, DMSO) 1.30 (3H, t, J = 7.1 Hz, CH$_3$), 2.72 (2H, q, J = 7.3 Hz, CH$_2$), 3.18 (4H, t, J = 7.3 Hz, CH$_2$), 3.66 (4H, t, J = 7.3 Hz, CH$_2$), 7.33-7.63 ppm (5H, m); ^{13}C NMR (δ ppm, DMSO) 12.86 (CH$_3$), 21.28 (CH$_2$), 50.18 (CH$_2$), 66.32 (CH$_2$), 80.71 (C-4a), 115.12 (C-7a), 153.52 (C-5), 157.26 (C-4), 123.85–138.89 (C-arom); GC-MS: m/z(%) 283 (100), 213 (25), 147 (36). C$_{16}$H$_{18}$N$_6$O: Calculated (%): C 61.92, H 5.85, N 27.08, O 5.16 Found (%): C 61.90, H 5.83, N 27.10, O 5.17.

General procedure for synthesis of pyrazolopyrimidinones (7)

5-amino-4-cyano-1-substituted pyrazole 4 (10 mmol) was added portionwise over 1 h to a mildly refluxing formic acid solution (20 mL) containing concentrated sulphuric acid (1.2 mL). After an additional 30 min, the solution was cooled to 0°C and poured on crushed

ice. The resulting precipitate was collected by filtration, washed with water, and dried to give pyrazolopyrimidinones **7**.

1-phenyl-1H-*pyrazolo[3,4-d]pyrimidin-4(5H)-one (7a)*

Yield 82% mp = 164°C [methanol]; IR (cm^{-1}) 1663 (C = O), 1583 (C=N), 1545 (C=N), 1500 (C=C); NMR ^{1}H (δ ppm, DMSO) 7.34-7.65 (5H, m);7.72 (1H, s, H$_3$); 7.75 (1H, s, H$_6$); 8.01 ppm (1H, s, NH); ^{13}C NMR (δ ppm, DMSO): 93.01 (C-3a), 131.15 (C-7a), 136.63 (C-3), 142.00 (C-6), 159.87 (C-4), 124.54–129.33 (C-arom); GC-MS: m/z (%) 213 (100), 168 (28), 102 (58); C$_{11}$H$_8$N$_4$O: Calculated (%): C 62.26, H 3.80, N 26.40, O 7.54 Found (%): C 62.28, H 3.81, N 26.42, O 7.55.

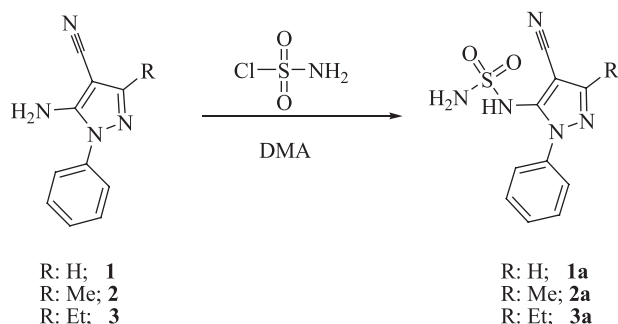### 3-Methyl-1-phenyl-1H-*pyrazolo[3,4-d]pyrimidin-4(5H)-one (7b)*

Yield: 86%. mp = 159°C [methanol]. IR (cm^{-1}): 1665 (C=O); 1580 (C=N); 1541 (C=N); 1506 (C=C); NMR ^{1}H (δ ppm, DMSO): 2.25 (3H, s, CH$_3$), 7.34-7.68 (5H, m), 7.72 (1H, s, H$_6$); 8.00 ppm (1H, s, NH); ^{13}C NMR (δ ppm, DMSO) 13.76 (CH$_3$), 93.00 (C-3a), 135.55 (C-3), 135.55 (C-7a), 139.76 (C-6), 160.30 (C-4), 124.54–129.33 (C-arom); GC-MS: m/z (%) 227 (100), 200 (36), 182 (12), 168 (50), 131 (33), 116 (44), 104 (20).

3-Ethyl-1-phenyl-1H-*pyrazolo[3,4-d]pyrimidin-4(5H)-one (7c)*

Yield 90%; mp = 168°C [methanol]; IR (cm^{-1}) 1665 (C = O), 1580 (C = N), 1541 (C = N), 1506 (C=C); NMR ^{1}H (δ ppm, DMSO) 3.15 (3H, t, *J* = 7.1 Hz, CH$_3$); 3.64 (2H, q, *J* = 7.1 Hz, CH$_2$); 7.34–7.60 (5H, m, CH = C); 7.78 (1H, s, H$_6$); 8.05 ppm (1H, s, NH); ^{13}C NMR (δ ppm, DMSO): C$_2$ 93.01 (C-3a); C$_3$ 131.15 (C-7a); C$_1$ 136.63 (C-3); C$_5$ 145.76 (C-6); C$_4$ 159.87 (C-4); 124.54–129.33 (C-arom); GC-MS m/z (%) = 241 (100), 186 (60), 141 (45), C$_{13}$H$_{12}$N$_4$O: Calculated (%): C 64.99, H 5.03, N 23.32, O 6.66 Found (%): C 64.97; H 5.00; N 23.35; O 6.65.

General procedure for the synthesis of compounds (8a–c)[47]

Scheme 3 shows the preparation of sulfamides **8a–c**. Freshly prepared sulfamoyl chloride (2.0 eq) was added to a 2.0 M solution of 5-amino-1-phenyl-1*H*-pyrazole-4-carbonitrile **4a**–**c** in dry DMA under a nitrogen atmosphere

and the solution was stirred at r.t. until starting material was consumed (TLC monitoring). Then the solution was quenched with slush and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with H$_2$O (4 × 20 mL), brine (3 × 20 mL) dried over Na$_2$SO$_4$, filtered and concentrated *in vacuo* to give a sticky residue that was purified to afford the desired sulfamides **8a**–**c** as white solids.

Synthesis of 1-phenyl-5-sulphamido-1H-*pyrazole-4-carbonitrile (8a)*

5-Amino-1-phenyl-1*H*-pyrazole-4-carbonitrile **4a** (0.05 g, 1.0 eq) was treated according to the general procedure previously described. The obtained residue was purified by silica gel column chromatography eluting with 50% ethyl acetate/*n*-hexane to afford **8a** as a white solid (Figure 1).

1-Phenyl-5-sulphamido-1*H*-pyrazole-4-carbonitrile **8a**: 54% yield; silica gel TLC R_f 0.09 (50% ethyl acetate/*n*-hexane); δ$_H$ (400 MHz, DMSO-d_6) 9.60 (1H, brs, exchange with D$_2$O, N*H*), 7.60 (8H, m, Ar-H, 3-H, SO$_2$N*H*$_2$), 7.42 (2H, s, exchange with D$_2$O, SO$_2$N*H*$_2$); δ$_C$ (100 MHz, DMSO-d_6) 155.5, 154.1, 141.3, 130.2, 125.0, 120.0, 117.0, 79.8.

Synthesis of 3-methyl-1-phenyl-5-sulphamido-1H-*pyrazole-4-carbonitrile 8b*

5-Amino-3-methyl-1-phenyl-1*H*-pyrazole-4-carbonitrile **4b** (0.05 g, 1.0 eq) was treated according to the general procedure previously described. The obtained residue was purified by silica gel column chromatography eluting with 70% ethyl acetate/*n*-hexane to afford **8b** as a white solid (Figure 2).

3-Methyl-1-phenyl-5-sulphamido-1*H*-pyrazole-4-carbonitrile **8b**: 48% yield; silica gel TLC R_f 0.11 (50% ethyl acetate/*n*-hexane); δ$_H$ (400 MHz, DMSO-d_6) 9.99 (1H, brs, exchange with D$_2$O, N*H*), 7.53 (5H, m, Ar-H), 7.42 (2H, s, exchange with D$_2$O, SO$_2$N*H*$_2$), 2.18 (3H, s

Scheme 3. Preparation of sulfamides **8a–c.**

R: H; **1**
R: Me; **2**
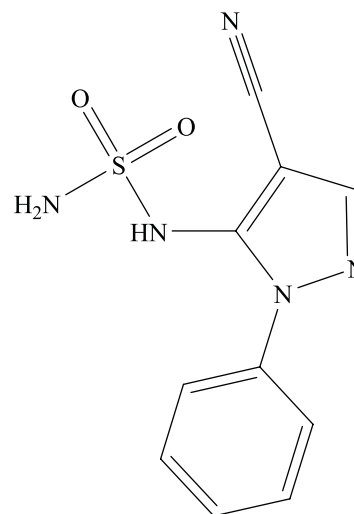R: Et; **3**
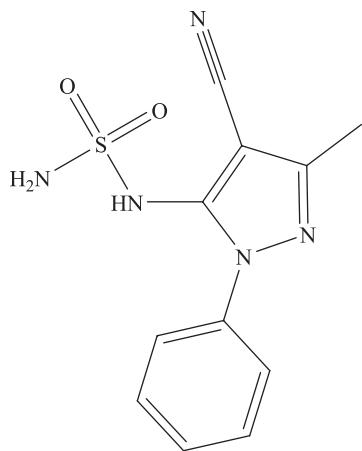
R: H; **1a**
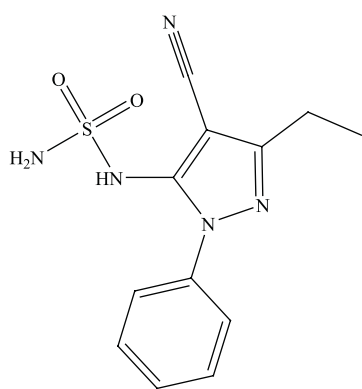R: Me; **2a**
R: Et; **3a**

Figure 1.

Figure 2.

Figure 3.

CH_3); δ_C (100 MHz, DMSO-d_6) 156.0, 155.1, 141.0, 130.1, 125.2, 119.0, 117.2, 80.0, 14.9.

Synthesis of 3-ethyl-1-phenyl-5-sulphamido-1H-*pyrazole-4-carbonitrile (8c)*

5-Amino-3-ethyl-1-phenyl-1*H*-pyrazole-4-carbonitrile **4c** (0.05 g, 1.0 eq) was treated according to the general procedure previously described. The obtained residue was purified by silica gel column chromatography eluting with 70% ethyl acetate/*n*-hexane to afford **8c** as a white solid (Figure 3).

3-Ethyl-1-phenyl-5-sulphamido-1*H*-pyrazole-4-carbonitrile **8c**: 52% yield; silica gel TLC R_f 0.13 (50% ethyl acetate/*n*-hexane); δ_H (400 MHz, DMSO-d_6) 9.98 (1H, brs, exchange with D$_2$O, NH), 7.66 (2H, d, J 9.6, Ar-H), 7.54 (3H, m, Ar-H), 7.41 (2H, s, exchange with D$_2$O, SO$_2$NH_2), 4.10 (2H, q, J 7.2, CH_2), 1.21 (3H, t, J = 7.2 Hz, CH_3); δ_C (100 MHz, DMSO-d_6) 157.2, 155.0, 141.0, 130.2, 125.1, 119.0, 117.4, 79.8, 23.9, 14.2.

CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO$_2$ hydration activity[41]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes/TRIS (pH 7.5 for

α-CAs, and 8.4, for β-CAs) as buffer, and 20 mM Na$_2$SO$_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO$_2$ hydration reaction for a period of 10–100 s[43]. The CO$_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (0.1 mM) were prepared in distilled-deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, as reported earlier[42], and represent the mean from at least three different determinations. All CA isofoms were recombinant ones obtained in house as reported earlier[43–45].

Declaration of interest

The authors declare no conflict of interest.

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