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Short communication

Synthesis and biological activities of pyrazolo[3,4-*g*]quinoxaline derivatives

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ABSTRACT

The synthesis of new pyrazolo[3,4-*g*]quinoxaline derivatives, as well as their Pim kinases (Pim-1, Pim-2, Pim-3) inhibitory potencies and *in vitro* antiproliferative activities toward a human fibroblast primary culture and three human solid cancer cell lines (PA1, PC3 and DU145) are described. The results obtained in this preliminary structure–activity relationship study have pointed out that most of the compounds in this series exhibited interesting *in vitro* Pim-3 kinase inhibitory potencies. Moreover, some of the tested compounds have demonstrated favorable antiproliferative potencies.

1. Introduction

As part of our ongoing studies focused on the preparation of potential biologically active compounds, and more particularly Pim kinases inhibitors, we were interested in the synthesis of new pyrazolo[3,4-*g*]quinoxaline derivatives. We have previously reported the preparation and biological activities of pyrrolocarbazole derivatives that have shown potent inhibitory potencies toward the Pim kinases [1,2]. These kinases have been described to play diverse biological roles in cell survival, proliferation and differentiation [1–3]. In this paper, we focus on the preparation of 1*H*-pyrazolo[3,4-*g*]quinoxaline derivatives by condensation of 1*H*-indazole-5,6-diamine with α-chloroketones and 1,2-diketones. To the best of our knowledge, there are only a few reports in the literature accounting for the synthesis of these derivatives. It has been shown that this family of compounds could display interesting biological activity, more particularly kinase inhibitory potency. Thus, pyrazolo[3,4-*g*]quinoxalines, substituted on the pyrazine moiety (C-6 and C-7 positions) by alkyl or aryl groups, were reported by Merck & Co. as Akt inhibitors [4–8]. Other derivatives were also described as tyrosine kinase or PKC inhibitors [9,10]. Two additional reports accounted for the synthesis of substituted pyrazolo[3,4-*g*]quinoxalines or pyrazolo[3,4-*g*]quinoxalin-4,9-diones [11,12]. Finally, this aromatic scaffold was rarely found incorporated into a more complex heteroaromatic structure [13–15]. Accordingly, we synthesized new pyrazolo[3,4-*g*]quinoxaline derivatives in which the pyrazine moiety was either substituted by ethyl groups or fused to a non-aromatic 5- or 6-membered ring. Then, the inhibitory potencies of these new compounds toward Pim-1, Pim-2 and Pim-3 kinases were evaluated. In addition, these pyrazoloquinoxaline derivatives were assayed for their *in vitro* antiproliferative activities toward a human fibroblast primary culture and three human solid cancer cell lines: PA1 (ovarian carcinoma), PC3 and DU145 (prostatic carcinoma).

2. Chemistry

The synthesis of compounds **11**–**17** was carried out starting from 1*H*-indazole-5,6-diamine **3**. The preparation of **3** was initially reported by Fries et al. from 6-nitroindazole **1** by nitration using H_2SO_4/HNO_3 and subsequent reduction with zinc dichloride in the presence of hydrochloric acid [16]. Starting from **1**, we applied other

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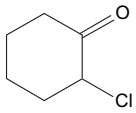
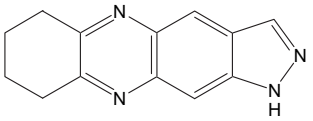
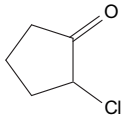
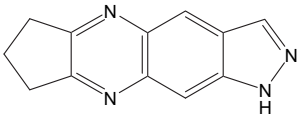
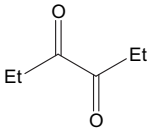
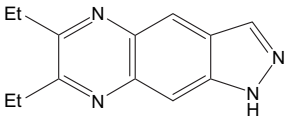
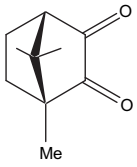
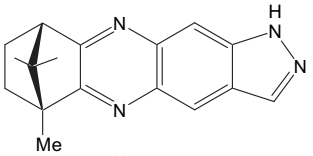
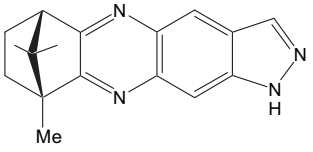
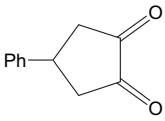
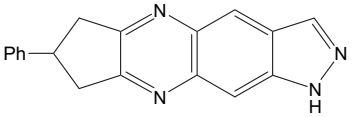
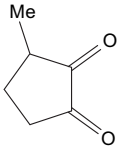
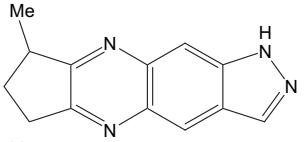
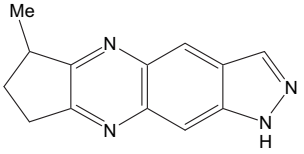
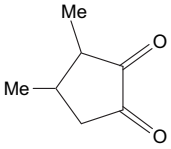
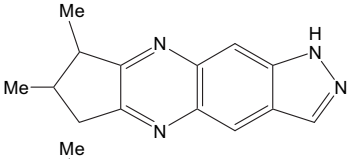
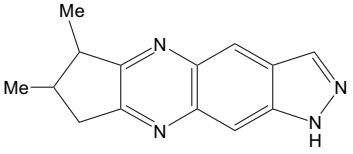
Table 1
Reaction conditions and yields for the preparation of compounds **11**–**17**. Conditions: (A) MeCN, PTSA; (B) THF, NEt_3; (C) AcOH.

1,2-Diketone or α-chloroketone		Conditions	Yield (%)	Product	
4		A	32	**11**	
5		B	22	**12**	
6		A	50	**13**	
7		A	35, 50	**14a**	
				14b	
8		C	33	**15**	
9		C	32	**16a**	
				16b	
10		C	30	**17a**	
				17b	

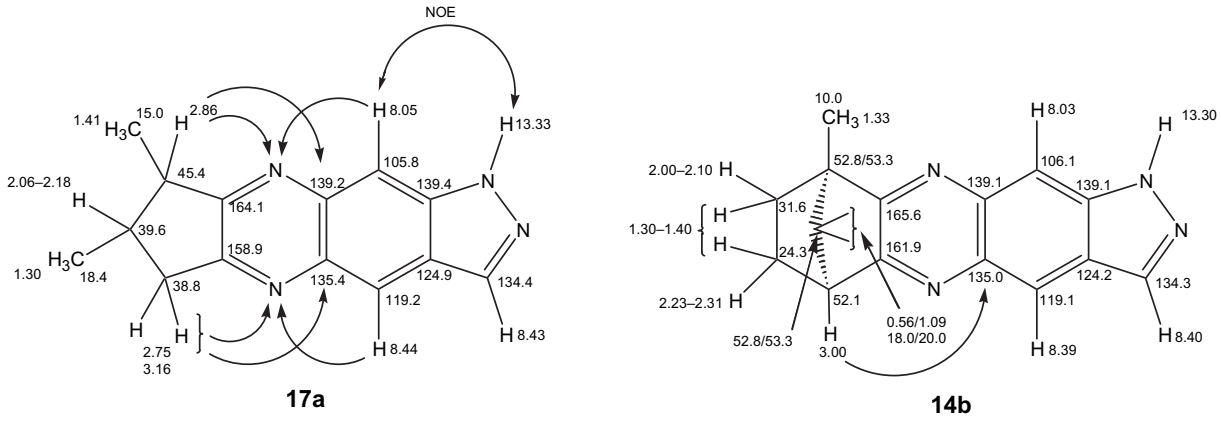
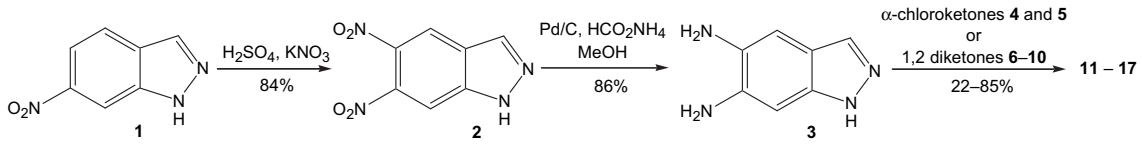


Fig. 1. ^{1}H and ^{13}C assignments of compounds **14b** and **17a**. Chemical shift values are given in ppm. Relevant $^{1}H-^{13}C$, $^{1}H-^{15}N$ and NOE correlations are indicated by arrows.

reaction conditions for the preparation of compound **3**, with a substantial yield improvement (Scheme 1). Nitration of commercially available 6-nitroindazole **1** was performed using H_2SO_4 and potassium nitrate [17] to give **2** in 84% yield after recrystallization from glacial acetic acid (in comparison, Fries et al., 70% [16]). Nitro groups were subsequently reduced using ammonium formate in the presence of 10% Pd/C to give **3** in 86% yield (in comparison, Fries et al., 70% [16]). Diamine **3** was further used for condensation reactions with various 1,2-diketones or α-chloroketones to give 1*H*-pyrazolo[3,4-*g*]quinoxaline derivatives **11**–**17** in moderate to good yields (22–85%) (Scheme 1, Table 1). Condensation with 2-chlorocyclohexanone **4** was performed in acetonitrile in the presence of *p*-toluenesulfonic acid (PTSA) as acidic catalyst [18] leading to **11** in 32% isolated yield. Unfortunately, when the same reaction conditions were applied to 2-chlorocyclopentanone **5**, the attempted product could not be obtained. Accordingly, the condensation of **5** with diamine **3** was achieved under basic conditions using triethylamine in THF to give **12** in 22% yield. The condensation reactions using 1,2-diketones **6**–**10** and diaminoindazole **3** were carried out in an acidic medium. The condensation with hexane-3,4-dione **6** was performed in acetonitrile in the presence of a catalytic amount of PTSA to give **13** in moderate yield. Using the same reaction procedure, the condensation with camphorquinone **7** led to the regioisomers **14a** and **14b** which were successfully separated by chromatography to give **14a** and **14b** in 35% and 50% yields, respectively. Unfortunately, the use of PTSA in acetonitrile with 5-membered ring diketones **8** [19,20], **9** and **10** led to degradation products. Therefore, the condensation reactions were performed in acetic acid according to literature procedure [21]. Thus, compounds **15**–**17** were obtained in 30–33% yields. As in the case of camphorquinone, a mixture of regioisomers was obtained when the condensation reactions were carried out with diones **9** and **10**. Despite our efforts, regioisomers **16a** and **16b** were not separable by chromatography. Fortunately, regioisomers **17a** and **17b** were partially isolated from the mixture.



Scheme 1. Synthesis of 1*H*-pyrazolo[3,4-*g*]quinoxaline derivatives **11**–**17**.

The structures of the regioisomers **14a**/**14b** and **17a**/**17b** were determined using 1D ^{1}H and ^{13}C NMR spectra and 2D NMR experiments ($^{1}H-^{13}C$ HSQC, $^{1}H-^{13}C$ HMBC and $^{1}H-^{15}N$ HMBC and

Table 2
Kinase inhibitory potencies (% of residual kinase activity) and antiproliferative activities of compounds **3**, **11**–**15**, **17a** and **17b** (IC_{50} in μM). na: not active. nd: compounds that caused cell proliferation inhibition less than 50% at 10 μM when tested toward PA1 and PC3 cells.

Compounds	Kinase inhibition – % of residual kinase activity						Antiproliferative activity (IC_{50} in μM)			
	Pim-1		Pim-2		Pim-3		Fibro	PA1	PC3	DU145
	10 μM	1 μM	10 μM	1 μM	10 μM	1 μM				
3	59 ± 7	95 ± 1	na	na	32 ± 3	69 ± 1	nd	nd	nd	nd
11	55.1 ± 0.3	90 ± 2	na	na	19.7 ± 0.2	58 ± 2	>50	16 ± 1	>50	>50
12	66.6 ± 0.8	95 ± 1	na	na	46.4 ± 0.2	81 ± 2	nd	nd	nd	nd
13	49 ± 13	89 ± 3	na	na	31 ± 8	84 ± 5	>50	6.5 ± 0.8	>50	>50
14a	45 ± 7	88 ± 5	76 ± 13	82 ± 20	37 ± 2	95 ± 4	nd	nd	nd	nd
14b	71 ± 5	96 ± 4	78 ± 9	na	43 ± 3	83 ± 12	nd	nd	nd	nd
15	83 ± 9	na	na	na	79 ± 3	95 ± 14	>50	4.1 ± 0.3	>50	35 ± 4
17a	69 ± 2	na	na	na	33 ± 3	73 ± 2	>50	4.7 ± 0.4	>50	>50
17b	59 ± 1	88.5 ± 0.5	na	na	44 ± 8	72 ± 3	>50	6.1 ± 0.5	>50	>50

NOESY) (Fig. 1). According to a NOESY experiment performed with one of the regioisomers **17** (finally identified as **17a**), an NOE correlation was observed between the two hydrogens at 8.05 ppm and 13.33 ppm, leading in turn to the assignment of indazole moiety hydrogens at 8.43 ppm and 8.44 ppm. Furthermore, $^{1}H-^{15}N$ HMBC experiment led to the observation of relevant ^{3}J correlations between two aromatic hydrogens (8.05, 8.44 ppm), three aliphatic hydrogens (2.86 ppm, 2.75 ppm, 3.16 ppm) and the two nitrogens of the pyrazine moiety allowing to unambiguous identification of the regioisomer **17a**. Final assignments were made from $^{1}H-^{13}C$ HMBC experiments based on the correlations observed between the hydrogens at 2.86, 2.75 and 3.16 ppm and the aromatic carbons at 135.4 and 139.2 ppm.

Regarding regioisomers **14a**/**14b**, $^{1}H-^{15}N$ HMBC experiments carried out on one of the regioisomers **14** did not lead to the observation of correlations between aromatic or aliphatic hydrogens and the two pyrazine nitrogens. Fortunately, long range $^{1}H-^{13}C$ coupling was observed between the hydrogen at 3.00 ppm and a carbon at 135.0 ppm. Based on the NMR assignments determined for compound **17a** (particularly the long range coupling between the hydrogens at 2.75 ppm and 3.16 ppm and the equivalent carbon at 135.4 ppm) the studied regioisomer was identified as **14b**. Final assignments were performed according to the $^{1}H-^{13}C$ HSQC and $^{1}H-^{13}C$ HMBC experiments.

3. Results and discussion

The biological activities (kinase inhibitory potency and *in vitro* antiproliferative activity) were determined for compounds **11**–**15**, **17a** and **17b**. Due to purification problems, isomers **16a** and **16b** were not evaluated for their biological properties.

The kinase inhibitory potencies of compounds **11**–**15**, **17a** and **17b** were evaluated in duplicate as already described in the literature by Cohen's group [22]. The percentages of residual activity, when the compounds were tested at 10 μM and 1 μM toward Pim kinases (Pim-1, Pim-2 and Pim-3), are reported in Table 2. According to these results, Pim-3 was the most inhibited protein kinase. For all compounds, except **15**, the percentages of Pim-3 residual activity were inferior to 50% when the compounds were tested at 10 μM. The most active compound toward Pim-3 was the cyclohexyl derivative **11** which induced 80% Pim-3 inhibition at 10 μM. Regarding Pim-1, the best inhibitory activities were found for compounds **3**, **11**, **13**, **14a** and **17b** with a percentage of residual activity in the range of 45–60% when tested at 10 μM. Finally, none of the tested compounds have shown any significant inhibitory potency toward Pim-2 (% of residual kinase activity > 75% when tested at 10 μM).

In vitro antiproliferative activities of compounds **11**–**15**, **17a** and **17b** were evaluated toward a human fibroblast primary culture and three human solid cancer cell lines: PA1 (ovarian carcinoma), PC3 and DU145 (prostatic carcinoma). All the compounds were firstly tested toward PA1 and PC3 at 1 μM and 10 μM, and IC_{50} values were only determined when the growth inhibition was found to be more than 50% with compounds tested at 10 μM. The antiproliferative effect of the tested drug was assessed by the resazurin reduction test [23]. Under the conditions used, none of the tested compounds have shown any significant antiproliferative activities toward the fibroblast and the prostatic carcinoma cells used (PC3, DU145) (Table 2). Conversely, compounds **13**, **15**, **17a** and **17b**, substituted at the C-6 and C-7 positions of pyrazolo[3,4-*g*]quinoxaline nucleus by ethyl groups or fused at the same positions to 5-membered rings, have demonstrated interesting inhibitory potencies toward PA1 cells, with IC_{50} values in the micromolar range. Moreover, these compounds have shown a favorable selectivity index for PA1 cells compared to the fibroblast primary culture. Interestingly, compound **12**, bearing a non-substituted 5-membered ring does not show any significant activity toward PA-1 cells, showing that the substitution of the non-aromatic ring plays an important role for the cytotoxic potency in this series.

In conclusion, we have described the synthesis of new pyrazolo [3,4-*g*]quinoxaline derivatives substituted at the C-6 and C-7 positions of the pyrazoloquinoxaline nucleus either by ethyl groups or fused on the pyrazine moiety to a non-aromatic 5- or 6-membered ring. The results obtained in this preliminary structure–activity relationship study have pointed out that in this series, except for compound **15**, all the derivatives exhibited interesting *in vitro* Pim-3 kinase inhibitory potencies. Moreover, some of the tested compounds have demonstrated favorable antiproliferative potencies. This suggests that these scaffolds could be of interest for the development of new Pim kinase inhibitors with antiproliferative activities. The preparation of a larger library of analogues is currently under investigation in our group.

4. Experimental

4.1. Chemistry

4.1.1. General

IR spectra were recorded on Shimadzu FTIR-8400S or Perkin–Elmer Spectrum 65 spectrometers ($\bar{\nu}$ in cm^{-1}). NMR spectra were performed on a Bruker AVANCE 400 (^{1}H: 400 MHz, ^{13}C: 100 MHz) or Bruker AVANCE 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz), chemical shifts δ in ppm, the following abbreviations are used: singlet (s), doublet (d), triplet (t), quadruplet (q), quintuplet (quint), doublet of doublet (dd), multiplet (m), broad signal (br s). Mass spectra (ESI+) were determined on a high-resolution Micro Q-Tof apparatus (CRMP, Université Blaise Pascal, Clermont-Ferrand, France). Chromatographic purifications were performed by flash silica gel Geduran SI 60 (Merck) 0.040–0.063 mm column chromatography. Reactions were monitored by TLC using fluorescent silica gel plates (60 F254 from Merck). Melting points were measured on a Reichert microscope and are uncorrected.

4.1.2. 5,6-Dinitro-1H-indazole **2**

A mixture of 6-nitro-1*H*-indazole **1** (5 g, 31 mmol) in concentrated sulfuric acid (70 mL) was cooled to 0 °C and was slowly added into a stirred solution of potassium nitrate (3.44 g, 34 mmol) in concentrated sulfuric acid (30 mL) at 0 °C. The solution was stirred for 16 h at room temperature and then was poured on ice (800 g). The solid was filtered off, washed with water and recrystallized from glacial acetic acid (50 mL) to give **2** (5.35 g, 26 mmol, 84%) as a yellow solid.

^{1}H NMR (400 MHz, DMSO-d_6): 8.45 (1H, s), 8.56 (1H, s), 8.84 (1H, s), 13.01 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 109.1, 121.3, 137.2 (CH_{arom}), 122.2, 135.8, 138.4, 141.6 (C_{arom}).

4.1.3. 1H-indazole-5,6-diamine **3**

To a mixture of **2** (200 mg, 0.96 mmol) and 10% Pd/C (200 mg, 0.19 mmol, 20 mol%) in methanol (6 mL) was added ammonium formate (600 mg, 9.5 mmol). The mixture was refluxed for 1 h and the catalyst was removed by filtration through a Celite pad, which was subsequently washed with methanol (30 mL). The filtrate was evaporated under reduced pressure to give **3** (122 mg, 0.82 mmol, 86%) as a grey solid. Compound **3** was used directly for the next step without any further purification.

^{1}H NMR (400 MHz, DMSO-d_6): 4.29 (2H, br s, NH_2), 4.80 (2H, br s, NH_2), 6.57 (1H, s), 6.72 (1H, s), 7.53 (1H, s), 12.02 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 91.6, 101.7, 131.3 (CH_{arom}), 115.8, 132.1, 136.3, 138.4 (C_{arom}).

4.1.4. 6,7,8,9-Tetrahydro-1H-pyrazolo[3,4-b]phenazine ***11***

A solution of 1*H*-indazole-5,6-diamine **3** (100 mg, 0.67 mmol), 2-chlorocyclohexanone **4** (116 mg, 0.87 mmol) and PTSA monohydrate (10 mol%) in acetonitrile (8 mL) was refluxed for 2 h. Solvent was evaporated and the residue was dissolved in EtOAc (20 mL) and a saturated aqueous $NaHCO_3$ solution (10 mL) was added. The organic layer was separated and dried over anhydrous $MgSO_4$. The residue obtained upon evaporation was purified by flash chromatography (cyclohexane to EtOAc) to give **11** (48 mg, 0.21 mmol, 32%) as a brown solid.

Mp = 174–175 °C; HRMS (ESI+) calcd for $C_{13}H_{13}N_4$ $(M+H)^+$ 225.1140, found 225.1141; IR (ATR): 3179, 1634, 1568 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.94–2.00 (4H, m), 3.05–3.11 (4H, m), 7.99 (1H, s), 8.428 (1H, s), 8.433 (1H, s), 13.30 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 22.27, 22.32 (CH_2), 32.7, 32.9 (CH_2), 104.7, 118.8, 134.5 (CH_{arom}), 125.4, 134.9, 138.6, 139.5, 152.4, 154.7 (C_{arom}).

4.1.5. 1,6,7,8-Tetrahydrocyclopenta[b]pyrazolo[3,4-g]quinoxaline ***12***

A mixture of 1*H*-indazole-5,6-diamine **3** (100 mg, 0.67 mmol), triethylamine (0.19 mL, 138 mg, 1.36 mmol) and 2-chlorocyclopentanone **5** (76 μL, 90 mg, 0.76 mmol) in anhydrous THF (3 mL) was refluxed overnight. After evaporation of the solvent, water was added and the mixture was extracted with EtOAc. The assembled organic fractions were dried over $MgSO_4$, evaporated and the residue was purified by flash chromatography (cyclohexane/EtOAc, 60:40–0:100) to give **12** (30.8 mg, 0.15 mmol, 22%) as a light yellow solid.

Mp = 139–144 °C; HRMS (ESI+) calcd for $C_{12}H_{11}N_4$ $(M+H)^+$ 211.0984, found 211.0998; IR (ATR): 3468, 1635, 1597 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 2.22 (2H, quint, $J = 7.5$ Hz), 3.10 (2H, t, $J = 7.5$ Hz), 3.11 (2H, t, $J = 7.5$ Hz), 8.02 (1H, s), 8.43 (1H, s), 8.44 (1H, s), 13.31 (1H, se); ^{13}C NMR (100 MHz, DMSO-d_6): 20.9, 31.5, 31.8 (CH_2), 105.6, 119.2, 134.4 (CH_{arom}), 124.9, 135.1, 139.0, 139.4, 159.3, 161.5 (C_{arom}).

4.1.6. 6,7-Diethyl-1H-pyrazolo[3,4-g]quinoxaline ***13***

The same procedure as for the preparation of compound **11** was used with 1*H*-indazole-5,6-diamine **3** (142 mg, 0.96 mmol) and hexane-3,4-dione **6** (164 mg, 1.44 mmol). Flash chromatography (cyclohexane to EtOAc) provided **4** (108 mg, 0.48 mmol, 50%) as a yellow solid.

Mp = 147–149 °C; HRMS (ESI+) calcd for $C_{13}H_{15}N_4$ $(M+H)^+$ 227.1297 found 227.1293. IR (ATR): 3178, 1690, 1636, 1573 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.350 (3H, t, $J = 7.5$ Hz, CH_3), 1.354 (3H, t, $J = 7.5$, CH_3), 3.02 (2H, q, $J = 7.5$ Hz, CH_2), 3.03 (2H, q, $J = 7.5$ Hz, CH_2), 8.01 (1H, s), 8.42 (1H, s), 8.45 (1H, s), 13.30 (1H, br s, N*H*); ^{13}C NMR (100 MHz, DMSO-d_6): 11.4, 11.5 (CH_3), 27.3, 27.5 (CH_2), 105.1, 119.0, 134.4 (CH_{arom}), 125.2, 134.5, 138.4, 139.5, 155.3, 157.5 (C_{arom}).

4.1.7. (6R,9S)-6-Methyl-6,9-dimethylmethano-6,7,8,9-tetrahydro-1H-pyrazolo[3,4-b]phenazine ***14a*** *and (6S,9R)-9-methyl-6,9-dimethylmethano-6,7,8,9-tetrahydro-1H-pyrazolo[3,4-b]phenazine* ***14b***

The same procedure as for the preparation of compound **11** was used with 1*H*-indazole-5,6-diamine **3** (130 mg, 0.88 mmol) and (1*R*)-(−)-camphorquinone **7** (218 mg, 1.31 mmol). Flash chromatography (Et_2O/cyclohexane, 85:15) provided **14a** (85 mg, 0.31 mmol, 35%) and **14b** (122 mg, 0.44 mmol, 50%) as yellow solids.

4.1.7.1. Compound ***14a****.* Mp = 198–201 °C; HRMS (ESI+) calcd for $C_{17}H_{19}N_4$ $(M+H)^+$ 279.1610 found 279.1618; IR (ATR): 3155, 1647, 1615, 1588 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.56 (3H, s, CH_3), 1.09 (3H, s, CH_3), 1.33 (3H, s), 1.30–1.40 (2H, m), 2.00–2.10 (1H, m), 2.24–2.31 (1H, m), 3.01 (1H, d, $J = 4.5$ Hz), 8.00 (1H, s), 8.39 (1H, s), 8.42 (1H, s), 13.29 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 10.0, 18.0, 20.0 (CH_3), 24.3, 31.6 (CH_2), 52.3 (CH), 52.8, 53.0 (C), 106.0, 119.2, 134.3 (CH), 124.2, 135.0, 139.1 (2C) (C_{arom}), 163.6, 164.0 (C_{arom}).

4.1.7.2. Compound ***14b****.* Mp = 208–209 °C; HRMS (ESI+) calcd for $C_{17}H_{19}N_4$ $(M+H)^+$ 279.1610 found 279.1622; IR (ATR): 3161, 1650, 1616, 1587 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.56 (3H, s, CH_3), 1.09 (3H, s, CH_3), 1.33 (3H, s, CH_3), 1.30–1.40 (2H, m), 2.00–2.10 (1H, m), 2.23–2.31 (1H, m), 3.00 (1H, d, $J = 4.5$ Hz), 8.03 (1H, s), 8.39 (1H, s), 8.40 (1H, s), 13.30 (1H, br s, NH); ^{13}C NMR (100 MHz, DMSO-d_6): 10.0, 18.0, 20.0 (CH_3), 24.3, 31.6 (CH_2), 52.1 (CH), 52.8, 53.3 (C), 106.1, 119.1, 134.3 (CH_{arom}), 124.2, 135.0, 139.05, 139.13, 161.9, 165.6 (C_{arom}).

4.1.8. (±)-7-Phenyl-1,6,7,8-tetrahydrocyclopenta[b]pyrazolo[3,4-g]quinoxaline ***15***

To a solution of 1*H*-indazole-5,6-diamine **3** (12.6 mg, 0.085 mmol) in glacial acetic acid (0.3 mL) was added a solution of 4-phenylcyclopentane-1,2-dione **8** (14.8 mg, 0.085 mmol) in glacial acetic acid (0.3 mL). The mixture was stirred for 1 h in an oil bath heated at 80 °C. After cooling, the solution was poured into ice-water (10 mL) and then neutralized with a 5 M aqueous NaOH solution. After extraction with EtOAc, the assembled organic fractions were dried over $MgSO_4$ and evaporated. The brown oil was purified by flash chromatography (cyclohexane/EtOAc, 7:3) to give **15** (8 mg, 0.03 mmol, 33%) as a light yellow solid.

Mp >280 °C; HRMS (ESI+) calcd for $C_{18}H_{15}N_4$ $(M+H)^+$ 287.1297, found 287.1290; IR (ATR): 3190, 1644, 1607, 1494, 1355 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 3.25–3.33 (2H, m), 3.51 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 8.0$ Hz), 3.52 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 8.0$ Hz), 3.82–3.92 (1H, m), 7.27 (1H, t, $J = 7.5$ Hz), 7.37 (2H, t, $J = 7.5$ Hz), 7.45 (2H, d, $J = 7.5$ Hz), 8.06 (1H, s), 8.45 (1H, s), 8.48 (1H, s), 13.34 (1H, se); ^{13}C NMR (100 MHz, DMSO-d_6): 39.6 (CH_2), 40.8 (CH), 105.7, 119.4, 126.6, 127.1 (2C), 128.5 (2C), 134.5 (CH_{arom}), 125.0, 135.3, 139.2, 139.4, 143.7, 158.6, 160.8 (C_{arom}).

4.1.9. (±)-8-Methyl-1,6,7,8-tetrahydrocyclopenta[b]pyrazolo[3,4-g]quinoxaline ***16a*** *and (±)-6-methyl-1,6,7,8-tetrahydrocyclopenta[b]pyrazolo[3,4-g]quinoxaline* ***16b***

To a solution of 1*H*-indazole-5,6-diamine **3** (50 mg, 0.34 mmol) in glacial acetic acid (1.5 mL) was added 3-methylcyclopentane-1,2-dione **9** (37.8 mg, 0.34 mmol). The mixture was stirred for 30 min in an oil bath heated at 110 °C. After cooling, the solution was poured into ice-water (10 mL) and then neutralized with a 5 M aqueous NaOH solution. After extraction with EtOAc, the assembled organic fractions were dried over $MgSO_4$ and evaporated. The brown oil was purified by flash chromatography (cyclohexane/EtOAc, 7:3–2:8) to give a mixture of regioisomers **16a** and **16b** (24 mg, 0.11 mmol, 32%) as a light yellow solid. The regioisomers ratio (81:19) was determined from the ^1H NMR spectra on the signals at 8.03 ppm (minor isomer) and 8.06 ppm (major isomer).

HRMS (ESI+) calcd for $C_{13}H_{13}N_4$ $(M+H)^+$ 225.1140, found 225.1144. ^1H NMR (400 MHz, DMSO-d_6): 1.41 ($3H^a + 3H^b$, d, $J = 7.0$ Hz), 1.72–1.82 ($1H^a + 1H^b$, m), 3.04–3.10 ($2H^a + 2H^b$, m), 8.03 ($1H^b$, s), 8.06 ($1H^a$, s), 8.43 ($1H^a + 1H^b$, s), 8.45 ($1H^a$, s), 8.48 ($1H^b$, s), 13.28–13.35 ($1H^a + 1H^b$, se). ^{13}C NMR (100 MHz, DMSO-d_6), major regioisomer: 17.6 (CH_3), 30.2, 30.4 (CH_2), 37.9 (CH), 105.8, 119.2, 134.4 (CH_{arom}), 124.9, 135.2, 139.1, 139.4, 159.2, 164.1 (C_{arom}).

4.1.10. (±)-7,8-Dimethyl-1,6,7,8-tetrahydrocyclopenta[b]pyrazolo[3,4-g]quinoxaline ***17a*** *and (±)-6,7-dimethyl-1,6,7,8-tetrahydrocyclopenta[b]pyrazolo[3,4-g]quinoxaline* ***17b***

To a solution of 1*H*-indazole-5,6-diamine **3** (200 mg, 1.35 mmol) in glacial acetic acid (5 mL) was added 3,4-dimethylcyclopentane-

1,2-dione **10** (170 mg, 1.35 mmol). The mixture was stirred for 30 min in an oil bath heated at 80 °C. After cooling, the solution was poured into ice-water (40 mL) and then neutralized with a 5 M aqueous NaOH solution. After extraction with EtOAc, the assembled organic fractions were dried over $MgSO_4$ and evaporated. The brown oil was purified by flash chromatography (Et_2O/cyclohexane, 6:4) to give a mixture of regioisomers **17a** and **17b** (96 mg, 0.40 mmol, 30%) as a yellow solid. Another chromatography led to a partial separation of the mixture with **17a** (9.6 mg, 0.04 mmol, 3%) and **17b** (12 mg, 0.05 mmol, 4%) as yellow solids.

*4.1.10.1. Compound **17a**.* Mp = 183–189 °C; HRMS (ESI+) calcd for $C_{14}H_{15}N_4$ $(M+H)^+$ 239.1297, found 239.1303; IR (ATR): 3179, 1635, 1570 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.30 (3H, d, $J = 6.5$ Hz), 1.41 (3H, d, $J = 7.0$ Hz), 2.06–2.18 (1H, m), 2.75 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 10.5$ Hz), 2.86 (1H, dq, $J_1 = 10.0$ Hz, $J_2 = 7.0$ Hz), 3,16 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 7.5$ Hz), 8.05 (1H, s), 8.43 (1H, s), 8.44 (1H, s), 13.33 (1H, se); ^{13}C NMR (100 MHz, DMSO-d_6): 15.0, 18.4 (CH_3), 38.8 (CH_2), 39.6, 45.4 (CH), 105.8, 119.2, 134.4 (CH_{arom}), 124.9, 135.4, 139.2, 139.4, 158.9, 164.1 (C_{arom}).

*4.1.10.2. Compound **17b**.* Mp = 206–216 °C; HRMS (ESI+) calcd for $C_{14}H_{15}N_4$ $(M+H)^+$ 239.1297, found 239.1295; IR (ATR): 3142, 1638, 1601 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.30 (3H, d, $J = 6.5$ Hz), 1.41 (3H, d, $J = 7.0$ Hz), 2.06–2.18 (1H, m), 2.77 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 10.5$ Hz), 2.86 (1H, dq, $J_1 = 10.0$ Hz, $J_2 = 7.0$ Hz), 3.17 (1H, dd, $J_1 = 17.0$ Hz, $J_2 = 7.5$ Hz), 8.03 (1H, s), 8.42 (1H, s), 8.47 (1H, s), 13.31 (1H, se); ^{13}C NMR (100 MHz, DMSO-d_6): 15.1, 18.4 (CH_3), 39.0 (CH_2), 39.6, 45.1 (CH), 105.7, 119.4, 134.4 (CH_{arom}), 124.8, 135.3, 139.3, 139.4, 161.0, 161.9 (C_{arom}).

4.2. In vitro kinase inhibition assays

4.2.1. In vitro kinase inhibition assays

The procedures for the *in vitro* protein kinase assays and for the expression and activation of the protein kinases have been detailed previously [21].

4.2.2. Source and purification of kinases

All protein kinases were of human origin and encoded full-length proteins. All proteins were either expressed as GST (glutathione transferase) fusion proteins in Escherichia coli or as hexahistidine (His_6)-tagged proteins in Sf21 (*Spodoptera frugiperda* 21) insect cells. GST fusion proteins were purified by affinity chromatography on glutathione–Sepharose, and His_6-tagged proteins on nickel/nitrilotriacetate–agarose.

4.2.3. Protein kinase assays

All assays (25.5 μL volume) were carried out robotically at room temperature (21 °C) and were linear with respect to time and enzyme concentration under the conditions used. Assays were performed for 30 min using Multidrop Micro reagent dispensers (Thermo Electron Corporation, Waltham, MA, USA) in a 96-well format. The concentration of magnesium acetate in the assays was 10 mM and [γ-^{33}P]ATP (800 c.p.m./pmol) was used at 5 μM for Pim-2 and 20 μM for Pim-1 and Pim-3, in order to be at or below the K_m for ATP for each enzyme.

The assays were initiated with MgATP, stopped by the addition of 5 μL of 0.5 M orthophosphoric acid and spotted on to P81 filter plates using a unifilter harvester (Perkin–Elmer, Boston, MA, USA). Kinase substrates were RSRHSSYPAGT (300 μM) for Pim-1, Pim-2 and Pim-3. The enzymes were diluted in a buffer consisting of 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 1 mg/mL BSA and 0.1% 2-mercaptoethanol and assayed in a buffer comprising 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1% 2-mercaptoethanol.

The inhibition profile of the tested compounds was expressed as the percentage of the residual kinase activity for an inhibitor concentration of 1 or 10 μM. The IC_{50} values of inhibitors were determined after carrying out assays at ten different concentrations of each compound.

4.3. Antiproliferative activities

4.3.1. Cell cultures

Stock cell cultures were maintained as monolayers in 75-cm^2 culture flasks in Glutamax Eagle's minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal calf serum, 5 mL 100 mM sodium pyruvate, 5 mL of 100× non-essential amino acids and 2 mg gentamicin base. Cells were grown at 37 °C in a humidified incubator under an atmosphere containing 5% CO_2.

4.3.2. Survival assays

Cells were plated at a density of 5×10^3 cells in 150 μL culture medium in each well of 96-well microplates and were allowed to adhere for 16 h before treatment with tested drug. A stock solution 20 mM of each tested drug was prepared in DMSO and kept at −20 °C until use. Then 50 μL of each tested solution was added to the cultures. A 48 h continuous drug exposure protocol was used. The antiproliferative effect of the tested drug was assessed by the resazurin reduction test.

4.3.3. Resazurin reduction test

Plates were rinsed with 200 μL PBS at 37 °C and emptied by overturning on absorbent towelling. Then 150 μL of a 25 μg/mL solution of resazurin in MEM without phenol red was added to each well. Plates were incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. Fluorescence was then measured on an automated 96-well plate reader (Fluoroscan Ascent FL, Labsystem) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Under the conditions used, fluorescence was proportional to the number of living cells in the well. The IC_{50}, defined as the drug concentration required to inhibit cell proliferation by 50%, was calculated from the curve of concentration-dependent survival percentage, defined as fluorescence in experimental wells compared with fluorescence in control wells, after subtraction of the blank values.

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