Synthesis of *N*-aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines and their photodynamic properties in the human skin melanoma cell line G361

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Abstract

A small series of *N*-aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines was synthesized from easily accessible 1-phenyl-1*H*-pyrazol-3-ol *via* 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine and 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine intermediates and their subsequent use in palladium catalyzed Buchwald-Hartwig cross-coupling reaction with various anilines. Majority of the compounds were not significantly cytotoxic to melanoma G361 cells in the dark up to 10 μM concentration, but their activity could be increased by irradiation with visible blue light (414 nm). The most active compound **10** possessed EC_{50} values of 3.5, 1.6 and 0.9 μM in cells irradiated with 1, 5 and 10 J/cm^2, respectively. The treatment caused generation of reactive oxygen species in cells and extensive DNA damage, documented by the comet assay and by detection of phosphorylated histone H2A.X, followed by apoptotic cell death. Our results suggest that *N*-aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines could serve as a potential source of photosensitizing compounds with anticancer activities.

Keywords

apoptosis; Buchwald–Hartwig amination; cytotoxicity; DNA damage; photodynamic effect; pyrazole; reactive oxygen species

1. Introduction

Photodynamic therapy (PDT) uses by itself non-toxic medication – photosensitizer (PS), which upon activation by specific light source within target cells produces reactive oxygen species and causes cell destruction [1]. The main advantages of PDT over traditional treatments include low systemic toxicity, spatiotemporal selectivity and the possibility of undergoing repeated treatments without the development of resistance [2,3]. It is thus a promising treatment for various, especially oncological, diseases [4], including but not limited to skin basal cell carcinoma [5,6], oral [7], lung [8,9], cervical [10], breast [11], prostate [12], head and neck [13] cancers. Furthermore, it can be used for treating acne [14], macular degeneration [15], Barrett‘s esophagus [16], atherosclerosis [17] or inactivating pathogens [18–22].

Despite huge potential of PDT over classical treatments, up to date, only five photosensitizers have been commercialized and around fifteen more are in clinical trials [4,23]. The most researched photosensitizers are by far derivatives of porphyrins [24]. Besides them, other types of elongated

conjugated systems possessing compounds have also been reported to possess photodynamic properties, such as cyanines [25–27], BODIPYs [28–30], aza-BODIPYs [31], rhodamines [32,33], coumarines [34], squaraines [35], etc. While pyrazoles are acknowledged for their diverse variety of biological activities [36–38], only several studies address their potential use in photodynamic therapy. Pyrazole-fused porphyrins were prepared by Yang *et al.*, unfortunately, as of now their properties were not reported [39]. Several pyrazolo[3,4-*h*]quinolones were revealed to possess sub-micromolar phototoxicity and to photoinduce cell death without DNA damage in cancer cells [40]. Pereira *et al.* developed dihydroxymethyl-4,5,6,7-tetrahydropyrazolo[1,5-*a*]pyridine-fused *meso*-tetraphenylchlorin which showed nanomolar activity against melanoma cells which are typically resistant to photodynamic therapy [41]. We recently reported strong photodynamic effect in melanoma G361 cells possessing pyrazole–indole hybrid which induces cell death through the production of ROS and extensive DNA damage [42].

In our previous works, we also investigated synthesis and biological activities of various annulated pyrazole ring systems such as benzopyrano[2,3-*c*]pyrazol-4(2*H*)-ones [43], 2*H*-furo[2,3-*c*]pyrazoles [44], 2*H*-pyrazolo[4,3-*c*]pyridines [45,46], pyrazolo[4,3-*f*][1,2,3]triazolo[5,1-*c*][1,4]oxazepines [47], pyrazolo[4',3':3,4]pyrido[1,2-*a*]benzimidazoles [48], dipyrazolo[1,5-*a*:4',3'-*c*]pyridines [49], thieno[2,3-*c*]pyrazoles [50], 2,6-dihydropyrano[2,3-*c*]pyrazoles [51], pyrazolo[4′,3′:5,6]pyrano[4,3-*c*][1,2]oxazoles [52] and others. In continuation on our research on pyrazole derivatives, in this work we present synthesis of *N*-aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridines by Buchwald-Hartwig reaction and investigation of their photodynamic properties in the human skin melanoma cell line G361.

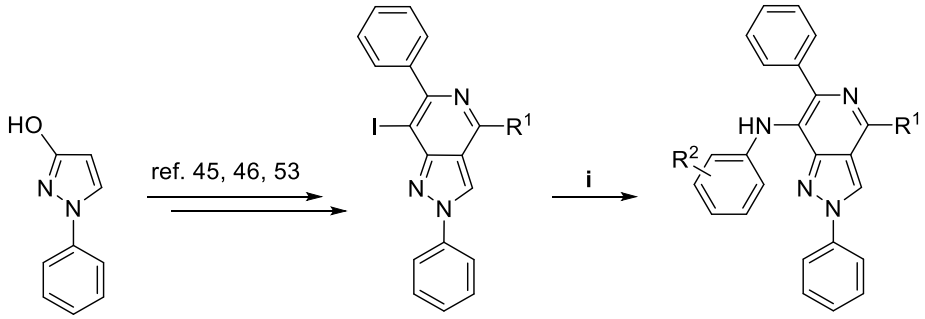
2. Results and discussion

2.1. Chemistry

To access a library of various *N*-aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines, intermediate 7-iodo-2,6-diphenyl-2*H*-pyrazol[4,3-*c*]pyridines **2-3** were prepared starting from 1-phenyl-1*H*-pyrazol-3-ol **1** *via* a multi-step synthetic sequence in accordance to our previously published procedures (Scheme S1, Supplementary File) [45,46,53]. In short, hydroxy group of 1-phenyl-1*H*-pyrazol-3-ol **1** was protected as a benzyl ether and a formyl group was introduced to 4- position by Vilsmeier–Haack reaction. After debenzylation with TFA, hydroxy group was

activated as a triflate and subsequently used in Sonogashira cross-coupling reaction with phenylacetylene to form 1-phenyl-3-(2-phenylethynyl)-1*H*-pyrazole-4-carbaldehyde. The obtained carbaldehyde was reduced to primary or secondary alcohols using either sodium borohydride as a reducing agent or methylmagnesium bromide as a Grignard reagent. The obtained alcohols were further converted into azides using $TMSN_3$ and catalytic amount of boron trifluoride diethyl etherate. Lastly, azides were treated with iodine and a proper base to form the pyrazolo[4,3-*c*]pyridine core with iodine in 7-position *via* electrophilic substitution reaction.

Prepared 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** and 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** were subsequently used in palladium catalysed Buchwald-Hartwig cross-coupling reaction adopting previously described reaction conditions [54]. 7-Iodopyrazolo[4,3-*c*]pyridines **2** and **3** were treated with an appropriate aniline, palladium acetate as a catalyst, 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl as a ligand and sodium *tert*-butoxide as a base in dioxane. To enhance the rate of the cross-coupling reactions, they were performed under microwave irradiation. The compounds **4-19** were obtained from iodo derivatives **2-3** in moderate to excellent yields (48-95%) (Table 1). Noteworthy, reactions were carried out under argon atmosphere and dry solvent due to the sensitivity of ligand and catalyst to air and moisture.



Scheme 1. Synthesis of compounds (**4-19**). *Reagents and conditions*: (i) R^2PhNH_2, $Pd(OAc)_2$, SPhos, NaO*t*Bu, dioxane, MW, 120 °C, 280 W, 1 h.

Table 1 Yields of new compounds **4-19** prepared from iodo derivatives **2-3**.

compound	R^1	R^2	Yield, %

4	H	H	92
5	H	4-MeO	48
6	H	3,4-diMeO	86
7	H	3,5-diMeO	60
8	H	2,4-diMeO	63
9	H	3,4,5-triMeO	95
10	H	2-CF_3O	56
11	H	3-CF_3O	56
12	Me	H	65
13	Me	4-MeO	77
14	Me	3,4-diMeO	60
15	Me	3,5-diMeO	49
16	Me	2,4-diMeO	73
17	Me	3,4,5-triMeO	63
18	Me	2-CF_3O	62
19	Me	3-CF_3O	82

The structures of compounds **4-19** were confirmed by NMR and IR spectroscopy, MS and HRMS spectrometry. The formation of amine can be quickly confirmed by IR spectrum in which peaks of amine group can be found at 3204-3404 cm^{-1}. Also, in comparison to starting materials **2** and **3**, the ^1H NMR spectra of all new derivatives have a broad NH singlet at 5.89-6.46 ppm, appropriate signals of the *N*-aryl protons at 5.97-7.21 ppm, while compounds **5-9** and **13-17** additionally have singlets of methoxy groups protons in the area of 3.65-3.83 ppm.

2.2. Optical properties

The fluorescence properties of final compounds **4-19** were briefly assessed in pH 7 Britton–Robinson buffer (Figure S1, Supplementary File). The excitation wavelength λ_{ex} was set to 380 nm. With an exception of 2,4-dimethoxyphenyl and 3,4,5-trimethoxyphenyl substituents at 7-position possessing compounds **8**, **9**, **16** and **17**, which had extremely low fluorescence intensity, the emission maxima λ_{em} of the remaining compounds were located in the 460-540 nm range,

which corresponds to the blue part of the visible light spectrum. Even though compounds absorbed both UV and visible blue light, to minimize negative effects of UV and near-UV light to the cells [55], a 414 nm emitting LED source was used for photodynamic experiments [56].

2.3. Biology

To assess photodynamic potency of the prepared compounds, G361 cells were treated with compounds for 4 h and then exposed to blue light (LED source, 414 nm), which was selected based on optical properties of the compounds (Figure S1, Supplementary File), at a total irradiation dose of 10 J/cm^2. The viability of the cells was quantified 72 h after irradiation by an MTT assay, revealing that most of the compounds possess photodynamic properties, with 4-unsubstituted derivatives **4-11** being typically more active than their 4-methyl counterparts **12-19**. However, there were few exceptions from this trend; 4-methyl substituted phenylamino and (3,5-dimethoxyphenyl)amino derivatives **12** and **15** were slightly more active than 4-unsubstituted homologs **4** and **7**. Compounds **5** and **10**, both of which lack methyl at position **4** and have either (4-methoxyphenyl)amino or [2-(trifluoromethoxy)phenyl]amino substituents at position **7**, respectively, were revealed to be the most potent photosensitizers with the lowest EC_{50} (1.1 μM). Noteworthy, with an exception of compound **8**, no dark toxicity was observed up to tested concentration (10 μM) (Table 1).

Table 2 EC_{50} values of compounds **4-19** in dark kept and light irradiated (414 nm, 10 J/cm^2) G361 cells.

compound	EC_{50} ± SD (μM)	
	dark	light energy (10 J/cm^2)
4	>10	2.7 ± 0.5
5	>10	1.1 ± 0.5
6	>10	1.3 ± 0.3
7	>10	8.0 ± 1.7
8	9.6 ± 0.2	3.1 ± 0.1
9	>10	>10
10	>10	1.1 ± 0.0
11	>10	1.8 ± 0.8

12	>10	2.1 ± 0.7
13	>10	8.8 ± 1.7
14	>10	7.1 ± 0.5
15	>10	4.5 ± 1.8
16	>10	9.9 ± 0.2
17	>10	>10
18	>10	5.3 ± 2.3
19	>10	2.0 ± 0.5

Subsequently, the viability of G361 cells upon treatment with one of the most potent compounds **10** was evaluated in a range of irradiation doses (1, 5, and 10 J/cm^2). As anticipated, the photoactivation proved to be light exposure-dependent. Namely, decreasing irradiation dose to 5 and 1 J/cm^2 increased the EC_{50} value to 1.6 and 3.5 μM, respectively. Conducting the experiment on human keratinocyte HaCaT cells revealed that these non-cancerous cells are approximately 2-fold less sensitive to the treatment, confirming the potential of compound **10** as a photodynamic agent (Table 3).

Table 3 Sensitivity of G361 and HaCaT cells to compound **10** in the dark and after exposure to various light doses (414 nm).

light energy (J/cm^2)	**EC_{50} ± SD (μM)**	
	G361	**HaCaT**
0	>10	>10
1	3.5 ± 0.3	5.7 ± 2.2
5	1.6 ± 0.4	1.9 ± 0.1
10	0.9 ± 0.3	2.0 ± 0.1

Generation of reactive oxygen species (ROS) is the key mechanism by which PDT causes localized cell death [57]. Therefore, to further assess the phototoxic activity of compound **10**, the production of ROS in treated G361 cells was quantified by using 2',7'-dichlorodihydrofluorescein diacetate. As expected, no ROS production was observed in untreated and/or only irradiated cells, while

combination of compound **10** in a range of tested concentrations (1.1, 3.3 and 10 µM) and irradiation doses (1, 5 and 10 J/cm^2) caused ROS generation (Figure 1).

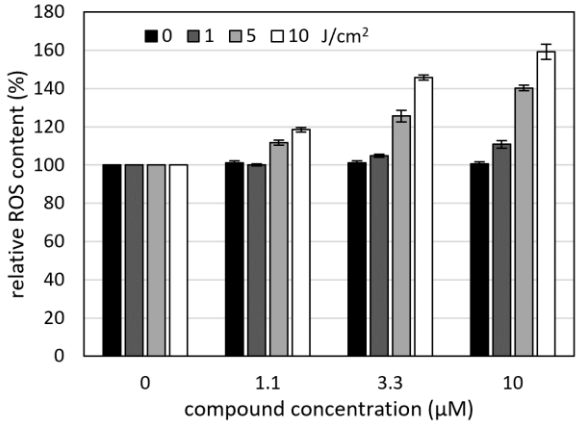


Figure 1 Production of reactive oxygen species in the G361 cells treated with compound **10** (1.1, 3.3 or 10 µM) in combination with light irradiation (414 nm).

Next, the analysis of cellular DNA content of compound **10** treated and irradiated G361 cells by flow cytometry revealed an increase of subdiploid populations in both concentration and light irradiation dependent manner (Table 4). This result demonstrates that apoptosis is a probable cause of cell death. Notably, at higher irradiation doses, a mild increase of subdiploid populations was also detected in compound-untreated cells, which is to be anticipated as high fluences irradiation with blue light (412–426 nm) is known to exert toxic effects in human skin cells [58]. Moreover, slightly increased subdiploid populations in cell cultures kept in the dark indicated that compound **10** has some toxic effect by itself, however this effect is low as documented by EC_{50} values >10 µM.

Table 4 Quantification (%) of subdiploid population of G361 cells treated with compound **10** in combination with light irradiation (414 nm).

concentration (µM)	**light energy (J/cm^2)**			
	0	1	5	10
0	3.4	5.0	4.4	6.1
1.1	4.6	5.4	4.5	9.7
3.3	5.0	5.0	15.0	70.0
10	5.9	5.3	63.3	88.3

To confirm apoptosis as a mechanism of cell death induced by irradiation in cells treated by compound **10**, activity of pro-apoptotic caspases 3 and 7, which are involved in mediating cell death signaling transduction, was quantified (Figure 2). The obtained results further confirmed initially observed capacity of compound **10** to induce apoptosis under light irradiation. Combining 10 µM concentration and 10 J/cm^2 irradiation, however, resulted in a severe cell death and no caspase 3/7 activity could be detected.

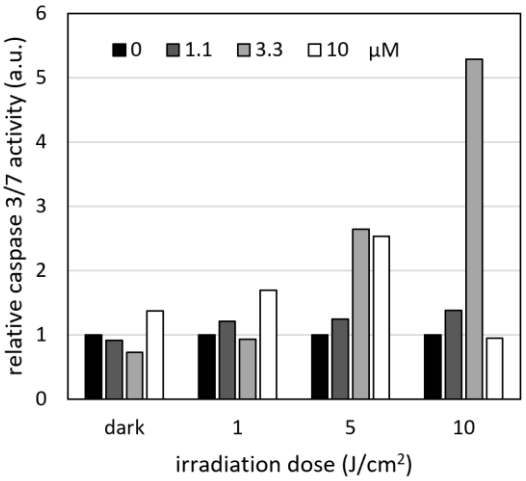


Figure 2 Relative caspase 3/7 activity in G361 cells treated with compound **10** (1.1, 3.3 or 10 µM) in combination with light irradiation (414 nm). The cells were treated with compound **10** for 4 h, irradiated (414 nm) and cultivated for further 24 h. After this period, the cells were harvested and the lysates were assayed for caspase 3/7 activity.

The compounds can kill cancer cells by inducing apoptotic cell death, leading to extensive DNA damage. In order to determine the extent of DNA fragmentation in G361 cells exposed to a combination of compound **10** and irradiation, a comet assay was performed. Percentual DNA content in the comet heads (nuclei) and tails were quantified. Expectedly, and in line with other performed experiments, compound **10** caused significant DNA fragmentation, which was dependent both on compound dose and light energy (Figure 3). The degree of DNA fragmentation in individual treatment combinations is available from Table S1, Supplementary File. Importantly, control cells (i.e. cells treated by **10** and kept in the dark or irradiated cells untreated with the compound) exhibited no marks of DNA damage.

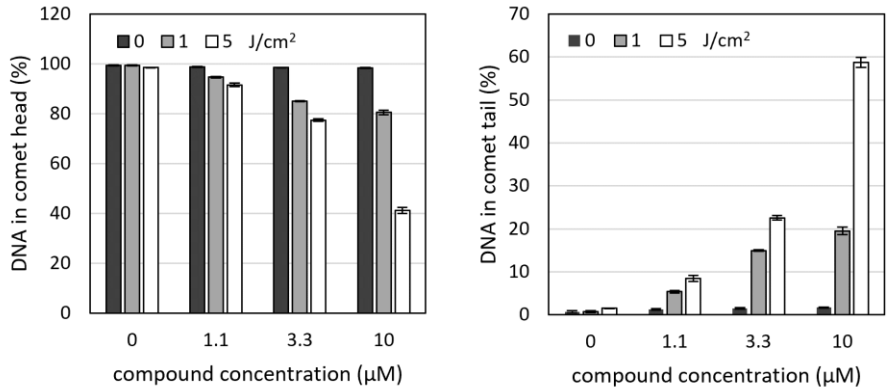


Figure 3. Percentual DNA content in the comet heads (nuclei) and tails of G361 cells treated with compound **10** (1.1, 3.3 or 10 µM) in combination with light irradiation (414 nm).

Finally, DNA damage and apoptotic cell death were independently confirmed by the means of immunoblotting analysis. G361 cells were treated with compound **10** at 1, 3 and 10 µM concentrations for 4 h, then either kept in the dark or exposed to light with a total irradiation dose of 1, 5 or 10 J/cm^2 followed by subsequent cultivation for 24 h. Increased levels of phosphorylated histone H2A.X at Ser-139, a marker of DNA damage, were observed when combining the higher concentrations of compound **10** and irradiation doses; the effect was the most apparent at 10 µM concentration and 5 J/cm^2 irradiation or at 3 µM concentration and 10 J/cm^2 irradiation (Figure 4). Analogously, the cleavage of PARP, an enzyme involved in DNA repair, which gets cut and inactivated by caspases during apoptotic cell death, was also apparent when combining said concentrations and irradiation doses. Finally, cleavage activation of caspase 7 further confirmed apoptosis. Absence of tubulin in the last lane (despite equal protein loading) could be attributed to severe cell death. Notably, the aforementioned changes could not be observed when the cells were kept in the dark or without exposure to compound **10**. It confirms that the above observation is a consequence of a photodynamic effect.

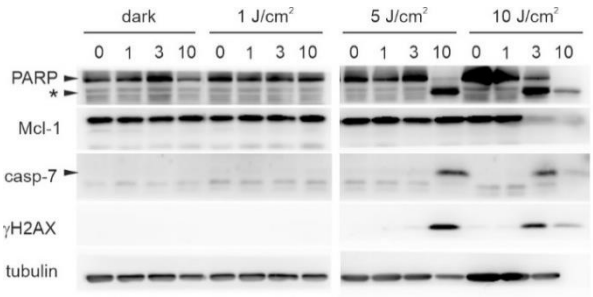


Figure 4 Light irradiation (414 nm) increases sensitivity of G361 cells to compound **10**. The cells were treated with 1, 3 or 10 μM compound for 4 h, irradiated and harvested 24 h after irradiation. Asterisk indicates 89 kDa cleavage fragment of PARP.

3. Conclusion

Synthesis of *N*-aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines was accomplished from easily accessible 1-phenyl-1*H*-pyrazol-3-ol *via* 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine and 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine intermediates *via* palladium catalysed Buchwald-Hartwig cross-coupling reaction with various anilines. Biological evaluation revealed that the prepared compounds exhibit very low dark cytotoxicity (>10 μM in most cases) but high photocytotoxicity to melanoma G361 cells under blue light (414 nm) irradiation. The most active compound **10** displays EC_{50} values of 3.5, 1.6 and 0.9 μM in cells irradiated with 1, 5 and 10 J/cm^2, respectively. Further experiments revealed that compound **10** causes cell death by producing ROS and damaging DNA. *N*-Aryl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amines could thus serve as a potential source of photosensitizing compounds with anticancer activities.

4. Materials and methods

4.1. Chemistry

4.1.1. General

All chemicals and solvents were purchased from commercial suppliers and used without further purification unless otherwise specified. The ^{1}H, ^{13}C and ^{15}N NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 solutions at 25 °C on either a Bruker Avance III 700 (700 MHz for ^{1}H, 176 MHz for ^{13}C, 71 MHz for ^{15}N) spectrometer equipped with a 5 mm TCI ^{1}H-^{13}C/^{15}N/D z-gradient cryoprobe or on Jeol ECA-500 (500 MHz for ^{1}H, 126 MHz for ^{13}C) spectrometer equipped with a 5 mm Royal probe. The chemical shifts, expressed in ppm, were relative to tetramethylsilane (TMS). The

^{15}N NMR spectra were referenced to neat, external nitromethane (coaxial capillary). FT-IR spectra were collected using the ATR method on a Bruker Vertex 70v spectrometer with an integrated Platinum ATR accessory or on a Bruker Tensor 27 spectrometer in KBr pellets. The melting points of crystalline compounds were determined in open capillary tubes with a Buchi M − 565 apparatus (temperature gradient – 2 °C/min) and are uncorrected. Mass spectra were recorded on Q-TOF MICRO spectrometer (Waters), analyses were performed in positive (ES^+) mode and molecular ions were recorded in $[M+H]^+$ forms. High-resolution mass spectrometry (HRMS) spectra were obtained in ESI mode on a Bruker MicrOTOF-Q III spectrometer. All reactions were performed in oven-dried flasks under an argon atmosphere with magnetic stirring. Reaction progress was monitored by TLC analysis on Macherey-Nagel™ ALUGRAM® Xtra SIL G/UV254 plates. TLC plates were visualized with UV light (wavelengths 254 and 365 nm) or iodine vapour. Compounds were purified by flash chromatography in a glass column (stationary phase – silica gel, high-purity grade 9385, pore size 60 Å, particle size - 230–400 mesh, supplier Sigma-Aldrich). ^{1}H, ^{13}C NMR spectra, as well as the HRMS data of new compounds, are provided in the Supplementary File.

4.1.2. Synthesis

4.1.2.1 General procedure for the synthesis of 7-substituted pyrazolo[4,3-*c*]pyridine derivatives 4-19 by Buchwald-Hartwig cross-coupling reaction with anilines

Appropriate 7-iodo-2*H*-pyrazolo[4,3-*c*]pyridine **2** or **3** (1 eq) was dissolved in dry dioxane. Then appropriate aniline (1.1 eq), NaO*t*Bu (1.6 eq), SPhos (0.3 eq) and $Pd(OAc)_2$ (0.1 eq) were added to the solution under argon atmosphere. The mixture was stirred at 120 °C under microwave irradiation (280 W, 300 Pa) for 1 h. Upon completion (monitored by TLC), the reaction mixture was cooled to room temperature, filtered through a pad of Celite and the filter cake was washed with EtOAc (20 mL). Filtrate was diluted with water (20 mL) and extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with brine (10 mL), dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography.

4.1.2.1.1. *N*,2,6-Triphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 4

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), aniline (0.015 mL, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol),

SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 50 mg (92%), orange crystals, mp = 234–235 °C, R_f = 0.11 (EtOAc/Hex, 1:3). ^{1}H NMR (700 MHz, $CDCl_3$): δ 6.17 (1H, s, NH), 6.80-6.89 (3H, m, NH-Ph 2,4,6-H), 7.10-7.17 (2H, m, NH-Ph 3,5-H), 7.24-7.29 (1H, m, C-Ph 4-H), 7.33-7.38 (2H, m, C-Ph 3,5-H), 7.41-7.45 (1H, m, N-Ph 4-H), 7.48-7.54 (2H, m, N-Ph 3,5-H), 7.69-7.77 (2H, m, C-Ph 2,6-H), 7.82-7.90 (2H, m, N-Ph 2,6-H), 8.59 (1H, s, 3-H), 9.07 (1H, s, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 117.4 (NH-Ph C-2,6), 120.5 (NH-Ph C-4), 121.1 (C-3a), 121.2 (N-Ph C-2,6), 121.6 (C-3), 124.3 (C-7), 127.6 (C-Ph C-4), 128.4 (C-Ph C-3,5), 128.5 (NH-Ph C-3,5), 128.7 (N-Ph C-4), 128.8 (C-Ph C-2,6), 129.7 (N-Ph C-3,5), 139.2 (C-6), 139.9 (C-Ph C-1), 140.8 (N-Ph C-1), 141.1 (C-4), 143.5 (NH-Ph C-1), 147.5 (C-7a). ^{15}N NMR (71 MHz, $CDCl_3$): δ −304.0 (NH), −147.3 (N-2), −100.5 (N-1), −78.1 (N-5). IR (ν, cm^{-1}): 3299 (NH), 3059, 3020 (CH_{arom}), 1601, 1590, 1497, 1426, 1346, 1202 (C=C, C=N, C–N), 751, 692, 684 (CH=CH of monosubstituted benzenes). MS (ES^+): *m/z* (%): 363 ($[M+H]^+$, 100). HRMS (ESI) for $C_{24}H_{19}N_4$ ($[M+H]^+$): calcd 363.1604, found 363.1606.

4.1.2.1.2. *N*-(4-Methoxyphenyl)-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 5

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 4-methoxyaniline (20 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 29 mg (48%), red crystals, mp = 135–136 °C, R_f = 0.08 (EtOAc/Hex, 1:3).^{1}H NMR (500 MHz, $CDCl_3$): δ 3.74 (3H, s, OCH_3) , 6.19 (1H, s, NH), 6.68-6.72 (2H, m, NH-Ph 3,5-H), 6.81-6.85 (2H, m, NH-Ph 2,6-H), 7.24-7.29 (1H, m, C-Ph 4-H), 7.33-7.37 (2H, m, C-Ph 3,5-H), 7.42-7.45 (1H, m, N-Ph 4-H), 7.50-7.54 (2H, m, N-Ph 3,5-H), 7.67-7.71 (2H, m, C-Ph 2,6-H), 7.83-7.87 (2H, m, N-Ph 2,6-H), 8.63 (1H, s, 3-H), 9.05 (1H, s, 4-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 55.7 (OCH_3), 113.9 (NH-Ph C-3,5), 120.3 (NH-Ph C-2,6), 120.8 (C-3a), 121.2 (N-Ph C-2,6), 122.2 (C-3), 126.2 (C-7), 127.8 (C-Ph C-4), 128.6 (C-Ph C-3,5), 128.8 (C-Ph C-2,6), 128.9 (N-Ph C-4), 129.8 (N-Ph C-3,5), 136.5 (NH-Ph C-1), 137.7 (C-6), 138.4 (C-Ph C-1), 139.3 (C-4), 139.9 (N-Ph C-1), 146.9 (C-7a), 154.8 (NH-Ph C-4). IR (ν, cm^{-1}): 3376 (NH), 3056, 3025 (CH_{arom}), 2923, 2852 (CH_{aliph}), 1595, 1505, 1464, 1234, 1212 (C=C, C=N, C–N), 1033 (C-O-C), 755, 699, 688 (CH=CH

of mono- and disubstituted benzenes).MS (ES^+): *m/z* (%): 393 ($[M+H]^+$, 100). HRMS (ESI) for $C_{25}H_{21}N_4O$ ($[M+H]^+$) calcd 393.1710, found 393.1710.

4.1.2.1.3. *N*-(3,4-Dimethoxyphenyl)-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 6

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 4-methoxyaniline (20 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 55 mg (86%), orange crystals, mp = 93–94 °C, R_f = 0.20 (EtOAc/Hex, 1:1). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.66 (3H, s, 3-OCH_3), 3.80 (3H, s, 4-OCH_3), 6.21 (1H, s, NH), 6.41-6.48 (2H, m, NH-Ph 2,6-H), 6.65-6.70 (1H, m, NH-Ph 3-H), 7.22-7.28 (1H, m, C-Ph 4-H), 7.31-7.36 (2H, m, C-Ph 3,5-H), 7.41-7.45 (1H, m, N-Ph 4-H), 7.49-7.54 (2H, m, N-Ph 3,5-H), 7.71-7.77 (2H, m, C-Ph 2,6-H), 7.83-7.90 (2H, m, N-Ph 2,6-H), 8.57 (1H, s, 3-H), 9.02 (1H, s, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 55.8 (3-OCH_3), 56.4 (4-OCH_3), 103.4 (NH-Ph C-2), 110.2 (NH-Ph C-6), 111.8 (NH-Ph C-5), 121.0 (C-3a), 121.2 (N-Ph C-2,6), 121.7 (C-3), 125.3 (C-7), 127.6 (C-Ph C-4), 128.5 (C-Ph C-3,5), 128.7 (N-Ph C-4), 128.8 (N-Ph C-2,6), 129.8 (N-Ph C-3,5), 137.3 (NH-Ph C-1), 139.0 (C-6), 139.6 (N-Ph C-1), 140.0 (N-Ph C-1), 140.1 (C-4), 143.8 (NH-Ph C-4), 147.3 (C-7a), 149.0 (NH-Ph C-3). ^{15}N NMR (71 MHz, $CDCl_3$): δ −306.4 (NH), −148.2 (N-2), −101.4 (N-1), −77.3 (N-5). IR (ν, cm^{-1}): 3363 (NH), 3055, 2995 (CH_{arom}), 2929, 2831 (CH_{aliph}), 1596, 1509, 1439, 1228 (C=C, C=N, C–N), 1024 (C-O-C), 756, 696 (CH=CH of mono- and disubstituted benzenes). MS (ES^+): *m/z* (%): 423 ($[M+H]^+$, 100). HRMS (ESI) for $C_{26}H_{23}N_4O_2$ ($[M+H]^+$) calcd 423.1816, found 423.1816.

4.1.2.1.4. *N*-(3,5-Dimethoxyphenyl)-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 7

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 3,5-dimethoxyaniline (25 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 38 mg (60%), brown crystals, mp = 140–141 °C, R_f = 0.28 (Hex:EtOAc 1:1). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.64 (3H, s, OCH_3), 5.98-6.01 (1H, m, NH-Ph 4-H), 6.01-6.05 (2H, m, NH-Ph 2,6-H), 6.17 (1H, s, NH), 7.27-7.30 (1H, m, C-Ph 4-H), 7.35-7.39 (2H, m, C-Ph 3,5-H), 7.42-7.45

(1H, m, N-Ph 4-H), 7.50-7.54 (2H, m, N-Ph 3,5-H), 7.74-7.78 (2H, m, C-Ph 2,6-H), 7.85-7.91 (2H, m, N-Ph 2,6-H), 8.58 (1H, s, 3-H), 9.08 (1H, s, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 55.2 (OCH_3), 93.2 (NH-Ph C-4), 95.7 (NH-Ph C-2,6), 121.0 (C-3a), 121.2 (N-Ph C-2,6), 121.8 (C-4), 124.0 (C-7), 127.7 (C-Ph C-4), 128.4 (C-Ph C-3,5), 128.70 (N-Ph C-4), 128.74 (C-Ph C-2,6), 129.7 (N-Ph C-3,5), 139.2 (C-Ph C-1), 139.9 (N-Ph C-1), 141.3 (C-6), 141.5 (C-4), 145.6 (NH-Ph C-1), 147.6 (C-7a), 161.1 (NH-Ph C-3,5). ^{15}N NMR (71 MHz, $CDCl_3$): −302.6 (NH), −146.9 (N-2), −101.7 (N-1), −79.3 (N-5). IR (ν, cm^{-1}): 3376 (NH), 3057, 2998 (CH_{arom}), 2933, 2837 (CH_{aliph}), 159, 1480, 1461, (C=C, C=N, C–N), 1199, 1148, 1054 (C-O-C), 756, 696, 686 (CH=CH of mono- and disubstituted benzenes). MS (ES$^+$): *m/z* (%): 423 ([M+H]$^+$, 99.7). HRMS (ESI) $C_{26}H_{23}N_4O_2$ ([M+H]$^+$) calcd 423.1816, found 423.1816.

4.1.2.1.5. *N*-(2,4-Dimethoxyphenyl)-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 8

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 2,4-dimethoxyaniline (25 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 40 mg (63%), brown crystals, mp = 108–110 °C, R_f = 0.28 (Hex:EtOAc 1:1). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.74 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 6.18-6.24 (1H, m, NH-Ph 4-H), 6.32 (1H, s, NH), 6.44-6.51 (1H, m, NH-Ph 3-H), 6.65-6.73 (1H, m, NH-Ph 6-H), 7.24-7.28 (1H, m, C-Ph 4-H), 7.32-7.37 (2H, m, C-Ph 3,5-H), 7.38-7.43 (1H, m, N-Ph 4-H), 7.47-7.53 (2H, m, N-Ph 3,5-H), 7.68-7.76 (2H, m, C-Ph 2,6-H), 7.83-7.91 (2H, m, N-Ph 2,6-H), 8.55 (1H, s, 3-H), 9.00 (1H, s, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 55.7 (2,4-OCH_3), 98.9 (NH-Ph C-3), 103.3 (NH-Ph C-5), 117.3 (NH-Ph C-6), 121.1 (C-3a), 121.2 (N-Ph C-2,6), 121.5 (C-3), 125.4 (C-7), 126.7 (NH-Ph C-1), 127.5 (C-Ph C-4), 128.4 (C-Ph C-3,5), 128.6 (N-Ph C-4), 128.9 (C-Ph C-2,6), 129.7 (N-Ph C-3,5), 139.6 (C-Ph C-1), 139.8 (C-6), 140.1 (C-4 and N-Ph C-1), 147.5 (C-7a), 150.1 (NH-Ph C-2), 154.4 (NH-Ph C-4). IR (ν, cm^{-1}): 3381 (NH), 3056, 2999 (CH_{arom}), 2932, 2832 (CH_{aliph}), 1595, 1512, 1463, 1283, 1203 (C=C, C=N, C–N), 1155, 1030 (C-O-C), 755, 699, 689 (CH=CH of mono- and disubstituted benzenes). MS (ES$^+$): *m/z* (%): 423 ([M+H]$^+$, 100). HRMS (ESI) $C_{26}H_{23}N_4O_2$ ([M+H]$^+$) calcd 423.1816, found 423.1816.

4.1.2.1.6. *N*-(3,4,5-Trimethoxyphenyl)-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 9

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 3,4,5-trimethoxyaniline (29 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 65 mg (95%), orange crystals, mp = 90–91 °C, R_f = 0.21 (Hex:EtOAc 1:1). ^{1}H NMR (700 MHz, $CDCl_3$): δ 3.65 (6H, s, 3,5-OCH_3), 3.75 (3H s, 4-OCH_3), 6.02-6.12 (2H, m, NH-Ph 2,6-H), 6.27 (1H, s, NH), 7.23-7.27 (1H, m, C-Ph 4-H), 7.32-7.37 (2H, m, C-Ph 3,5-H), 7.42-7.45 (1H, m, N-Ph 4-H), 7.50-7.55 (2H, m, N-Ph C-3,5), 7.72-7.79 (2H, m, C-Ph 2,6-H), 7.83-7.91 (2H, m, N-Ph 2,6-H), 8.57 (1H, s, 3-H), 9.04 (1H, s, 4-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 56.0 (3,5-OCH_3), 61.1 (OCH_3), 95.6 (NH-Ph C-2,6) 121.0 (C-3a), 121.2 (N-Ph C-2,6), 121.9 (C-3), 124.6 (C-7), 127.7 (C-Ph C-4), 128.5 (C-Ph C-3,5), 128.8 (C-Ph C-2,6), 128.9 (N-Ph C-4), 129.9 (N-Ph C-3,5), 132.4 (NH-Ph C-4), 139.4 (NH-Ph C-1), 139.6 (C-6 and C-Ph C-1), 140.0 (N-Ph C-1), 140.7 (C-4), 147.6 (C-7a), 153.2 (NH-Ph C-3,5). ^{15}N NMR (71 MHz, $CDCl_3$): δ −304.0 (NH), −147.3 (N-2), −101.7 (N-1), −76.9 (N-5). IR (ν, cm^{-1}): 3345 (NH), 3120, 3057 (CH_{arom}), 2930, 2838 (CH_{aliph}), 1595, 1501, 1463, 1409, 1231 (C=C, C=N, C–N), 1122, 1006 (C-O-C), 804, 756, 697, 689 (CH=CH of mono- and trisubstituted benzenes). MS (ES^+): *m/z* (%): 453 ($[M+H]^+$, 98). HRMS (ESI) $C_{27}H_{25}N_4O_3$ ($[M+H]^+$) calcd 453.1921, found 453.1921.

4.1.2.1.7. 2,6-Diphenyl-*N*-[2-(trifluoromethoxy)phenyl]-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 10

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 2-(trifluoromethoxy)aniline (28 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 38 mg (56%), yellow crystals, mp = 66–68 °C, R_f = 0.38 (Hex:EtOAc 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.46 (1H, s, NH), 6.67-6.73 (1H, m, NH-Ph 6-H), 6.75-6.79 (1H, m, NH-Ph 4-H), 6.86-6.91 (1H, m, NH-Ph 5-H), 7.17-7.21 (1H, m, NH-Ph 3-H), 7.24-7.28 (1H, m, C-Ph 4-H), 7.31-7.35 (2H, m, C-Ph 3,5-H), 7.44-7.48 (1H, m, N-Ph 4-H), 7.52-7.57 (2H, m, N-Ph 3,5-H), 7.72-7.76 (2H, m, C-Ph 2,6-H), 7.87-7.92 (2H, m, N-Ph 2,6-H), 8.68 (1H, s, 3-H), 9.18 (1H, s, 4-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 117.7 (NH-Ph C-6), 119.9, 120.9, 122.0 (CF_3, J = 126 Hz), 120.2 (NH-Ph C-4), 120.8 (NH-Ph C-3), 121.3 (N-Ph C-2,6), 122.5 (C-3), 123.5 (C-7), 126.6 (NH-

Ph C-5), 128.2 (C-Ph C-4), 128.6 (C-Ph C-3,5), 128.7 (C-Ph C-2,6), 129.1 (N-Ph C-4), 129.9 (N-Ph C-3,5), 135.7 (NH-Ph C-1), 138.3 (C-Ph C-1 and NH-Ph C-2), 139.9 (N-Ph C-1), 140.9 (C-6), 141.7 (C-4), 147.8 (C-7a). IR (ν, cm^{-1}): 3404 (NH), 3060, 3031 (CH_{arom}), 1606, 1501, 1484, 1244, 1213, 1165 (C=C, C=N, C–N, C-F), 751, 697, 687 (CH=CH of mono- and disubstituted benzenes). MS (ES^+): *m/z* (%): 447 ($[M+H]^+$, 97.8). HRMS (ESI) $C_{25}H_{18}F_3N_4O$ ($[M+H]^+$) calcd 447.1427, found 447.1427.

4.1.2.1.8. 2,6-Diphenyl-*N*-[3-(trifluoromethoxy)phenyl]-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 11

Prepared in accordance to general procedure from 7-iodo-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **2** (60 mg, 0.15 mmol), 3-(trifluoromethoxy)aniline (28 mg, 0.16 mmol), NaO*t*Bu (23 mg, 0.24 mmol), SPhos (18.6 mg, 0.045 mmol) and $Pd(OAc)_2$ (3.4 mg, 0.015 mmol) and dioxane (4.5 mL). The residue was purified by column chromatography (EtOAc/Hex, 1:3 to 1:2, v/v).

Yield: 38 mg (56%) orange crystals, mp = 77–78 °C, R_f = 0.38 (Hex:EtOAc 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.22 (1H, s, NH), 6.65-6.71 (2H, m, NH-Ph 4-H, 2-H or 6-H), 6.72-6.76 (1H, m, NH-Ph 2-H or 6-H), 7.09-7.16 (1H, m, NH-Ph 5-H), 7.28-7.32 (1H, m, C-Ph 4-H,), 7.36-7.40 (2H, m, C-Ph 3,5-H), 7.43-7.48 (1H, m, N-Ph 4-H), 7.51-7.56 (2H, m, N-Ph 3,5-H), 7.68-7.75 (2H, m, C-Ph 2,6-H), 7.84-7.90 (2H, m, N-Ph 2,6-H), 8.64 (1H, s, 3-H), 9.14 (1H, s, 4-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 109.7 (NH-Ph C-2 or C-6), 112.5 (NH-Ph C-5), 115.5 (NH-Ph C-2 or C-6), 119.5 (CF_3), 121.1 (C-3a), 121.4 (N-Ph C-2,6), 122.4 (C-3), 123.5 (C-7), 128.1 (C-Ph C-4), 128.7 (C-Ph C-3,5), 128.9 (C-Ph C-2,6), 129.0 (N-Ph C-4), 129.6 (NH-Ph C-5), 129.8 (N-Ph C-3,5), 138.6 (C-Ph C-1), 139.9 (N-Ph C-1), 141.6 (C-6), 142.0 (C-4), 145.2 (NH-Ph C-1), 147.5 (C-7a), 149.8 (NH-Ph C-3). IR (ν, cm^{-1}): 3204 (NH), 3061, 3048 (CH_{arom}), 1610, 1596, 1490, 1486, 1249, 1213, 1153 (C=C, C=N, C–N, C-F), 756, 695 (CH=CH of mono- and disubstituted benzenes). MS (ES^+): *m/z* (%): 447 ($[M+H]^+$, 100). HRMS (ESI) $C_{25}H_{18}F_3N_4O$ ($[M+H]^+$) calcd 447.1427, found 447.1427.

4.1.2.1.9 4-Methyl-*N*-2,6-triphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 12

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), aniline (0.012 mL, 0.13 mmol), NaO*t*Bu (18.7 mg,

0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 31 mg (65 %), mp = 165−169 °C. ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.87 (3H, s, CH_3), 5.94 (1H, s, NH), 6.81−6.83 (3H, m, NH-Ph 2,4,6-H), 7.13−7.15 (2H, m, NH-Ph 3,5-H), 7.26−7.29 (1H, m, C-Ph 4-H), 7.35−7.37 (2H, m, C-Ph 3,5-H), 7.40−7.42 (1H, m, N-Ph 4-H), 7.48−7.51 (2H, m, N-Ph 3,5-H), 7.72−7.73 (2H, m, C-Ph 2,6-H), 7.85−7.87 (2H, m, N-Ph 2,6-H), 8.57 (s, 1H, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 22.8 (CH_3), 117.0 (NH-Ph C-2,6), 120.1 (NH-Ph C-4), 121.20 (C-3a), 121.23 (N-Ph C-2,6), 122.0 (C-3), 122.4 (C-7), 127.8 (C-Ph C-4), 128.6 (C-Ph C-3,5), 128.65 (N-Ph C-4), 128.7 (NH-Ph C-3,5), 129.1 (C-Ph C-2,6), 129.7 (N-Ph C-3,5), 139.1 (C-6), 140.0 (N-Ph C-1), 141.6 (C-Ph C-1), 144.5 (NH-Ph C-1), 148.0 (C-7a), 150.4 (C-4). ^{15}N NMR (71 MHz, $CDCl_3$): δ −285.3 (NH), −160.2 (N-1), −148.3 (N-2), −82.2 (N-5). IR (KBr, ν, cm^{-1}): 3387 (NH), 3031, 3051 (CH_{arom}), 2985, 2914, 2850 (CH_{aliph}), 1598, 1511, 1499, 1310 (C=C, C=N, C−N), 760, 747, 696 (CH=CH of mono- and disubstituted benzenes). MS m/z (%): 377 ($[M+H]^+$, 100). HRMS (ESI) $C_{25}H_{21}N_4$ ($[M+H]^+$) calcd 377.1761, found 377.1763.

4.1.2.1.10 *N*-4-Methoxyphenyl-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 13

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 4-methoxyaniline (16 mg, 0.13 mmol), NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 38 mg (77%), light yellow crystals, mp = 89–90 °C, R_f = 0.11 (EtOAc/Hex, 1:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.86 (3H, s, CH_3), 3.74 (3H, s, OCH_3), 5.89 (1H, s, NH), 6.67-6.75 (2H, m, NH-Ph 3,5-H), 6.76-6.85 (2H, m, NH-Ph 2,6-H), 7.26-7.29 (1H, m, C-Ph 4-H), 7.32-7.38 (2H, m, C-Ph 3,5-H), 7.39-7.43 (1H, m, N-Ph 4-H), 7.48-7.53 (2H, m, N-Ph 3,5-H), 7.66-7.73 (2H, m, C-Ph 2,6-H), 7.81-7.88 (2H, m, N-Ph 2,6-H), 8.55 (1H, s, 3-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 22.8 (CH_3), 55.7 (OCH_3), 114.0 (NH-Ph C-2,6), 119.1 (NH-Ph C-3,5), 121.1 (N-Ph C-2,6), 121.2 (C-3a), 121.7 (C-3), 123.4 (C-7), 127.6 (C-Ph C-4), 128.5 (C-Ph C-3,5), 128.6 (N-Ph C-4), 129.0 (C-Ph C-2,6), 129.7 (N-Ph C-3,5), 138.0 (NH-Ph C-1), 139.3 (C-Ph C-1), 140.0 (N-Ph C-1), 140.3 (C-6), 147.6 (C-7a), 149.3 (C-4), 154.1 (NH-Ph C-4). IR (ν, cm^{-1}): 3375 (NH), 3053, 2994 (CH_{arom}), 2947, 2929, 2831 (CH_{aliph}), 1596, 1506, 1488, 1410, 1234, 1212 (C=C, C=N, C–N), 1029

(C-O-C), 756, 696, 689 (CH=CH of mono- and disubstituted benzenes). MS (ES^+): *m/z* (%): 407 ($[M+H]^+$, 100). HRMS (ESI) for $C_{26}H_{23}N_4O$ ($[M+H]^+$): calcd 407.1866, found 407.1866.

4.1.2.1.11 *N*-(3,4-Dimethoxyphenyl)-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 14

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 3,4-dimethoxyaniline (20 mg, 0.13 mmol), NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 32 mg (60%), brown crystals, mp = 202–203 °C, R_f = 0.08 (EtOAc/Hex, 1:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.88 (3H, s, CH_3), 3.67 (3H, s, 3-OCH_3), 3.81 (3H, s, 4-OCH_3), 5.98 (1H, s, NH), 6.38-6.43 (1H, m, NH-Ph 6-H), 6.43-6.46 (1H, m, NH-Ph 2-H), 6.65-6.72 (1H, m, NH-Ph 5-H), 7.23-7.29 (1H, m, C-Ph 4-H), 7.32-7.37 (2H, m, C-Ph 3,5-H), 7.41-7.45 (1H, m, N-Ph 4-H), 7.49-7.54 (2H, m N-Ph, 3,5-H), 7.70-7.75 (2H, m, C-Ph 2,6-H), 7.84-7.90 (2H, m, N-Ph 2,6-H), 8.57 (1H, s, 3-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 22.5 (CH_3), 55.8 (3-OCH_3), 56.4 (4-OCH_3), 103.0 (NH-Ph C-2), 109.6 (NH-Ph C-6), 111.8 (NH-Ph C-5), 121.0 (C-3a), 121.1 (N-Ph C-2,6), 122.0 (C-3), 123.5 (C-7), 127.8 (C-Ph C-4), 128.6 (C-Ph C-3,5), 128.7 (N-Ph C-4), 129.1 (C-Ph C-2,6), 129.8 (N-Ph C-3,5), 138.0 (NH-Ph C-1), 138.8 (N-Ph C-1), 139.4 (C-6), 139.9 (N-Ph C-1), 143.6 (NH-Ph C-4), 147.6 (C-7a), 149.1 (NH-Ph C-3), 149.3 (C-4). IR (ν, cm^{-1}): 3335 (NH), 3124, 3000 (CH_{arom}), 2951, 2931, 2830 (CH_{aliph}), 1595, 1526, 1507 1464, 1405, 1220, 1207 (C=C, C=N, C–N), 1153, 1027 (C-O-C), 759, 691 (CH=CH of mono- and trisubstituted benzenes). MS (ES^+): *m/z* (%): 437 ($[M+H]^+$, 100). HRMS (ESI) for $C_{27}H_{25}N_4O_2$ ($[M+H]^+$): calcd 437.1972, found 437.1972.

4.1.2.1.12 *N*-(3,5-Dimethoxyphenyl)-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 15

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 3,5-dimethoxyaniline (20 mg, 0.13 mmol), NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol)

and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 31 mg (49%), brown crystals, mp = 157–159 °C, R_f = 0.49 (Hex:EtOAc 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.89 (3H, s, CH_3), 3.65 (6H, s, OCH_3), 5.95 (1H, s, NH), 5.97-6.02 (3H, m, NH-Ph 2,5,6-H), 7.27-7.32 (1H, m, C-Ph 4-H), 7.35-7.40 (2H, m, C-Ph 3,5-H), 7.40-7.45 (1H, m, N-Ph 4-H), 7.49-7.54 (2H, m, N-Ph 3,5-H), 7.72-7.78 (2H, m, C-Ph 2,6-H), 7.85-7.91 (2H, m, N-Ph 2,6-H), 8.58 (1H, s, 3-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 22.7 (CH_3), 55.3 (OCH_3), 92.9 (NH-Ph C-4), 95.4 (NH-Ph C-2,6), 121.1 (C-3a), 121.3 (N-Ph C-2.6), 122.3 (C-3a and C-7), 128.0 (C-Ph C-4), 128.6 (C-Ph C-3.5), 128.8 (N-Ph C-4), 129.1 (C-Ph C-2,6), 129.8 (N-Ph C-3,5), 138.8 (C-Ph C-1), 140.0 (N-Ph C-1), 141.9 (C-6), 146.5 (NH-Ph C-1), 148.3 (C-7a), 151.0 (C-3), 161.2 (NH-Ph C-3,5). IR (ν, cm^{-1}): 3394, 3310 (NH), 3058, 3000 (CH_{arom}), 2920, 2851 (CH_{aliph}), 1595, 1516, 1459, 1416, 1378 (C=C, C=N, C–N), 1197, 1144, 1068, 1050 (C-O-C), 818, 763, 753, 688 (CH=CH of mono- and trisubstituted benzenes). MS (ES^+): *m/z* (%): 437 ($[M+H]^+$, 97.3). HRMS (ESI) $C_{27}H_{25}N_4O_2$ ($[M+H]^+$) calcd 437.1972, found 437.1972.

4.1.2.1.13 *N*-(2,4-Dimethoxyphenyl)-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 16

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 3,5-dimethoxyaniline (20 mg, 0.13 mmol), NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 39 mg (73%), orange crystals, mp = 92–95 °C, R_f = 0.44 (Hex:EtOAc 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.89 (3H, s, CH_3), 3.74 (3H, s, 4-OCH_3), 3.80 (3H, s, 3-OCH_3), 6.14 (1H, s, NH), 6.18-6.26 (1H, m, NH-Ph 5-H), 6.42-6.51 (1H, m, NH-Ph 3-H), 6.61-6.73 (1H, m, NH-Ph 6-H), 7.24-7.29 (1H, m, C-Ph 4-H), 7.31-7.37 (2H, m, C-Ph 3,5-H), 7.39-7.44 (1H, m, N-Ph 4-H), 7.48-7.54 (2H, m, N-Ph 3,5-H), 7.64-7.74 (2H, m, C-Ph 2,6-H), 7.83-7.94 (2H, m, N-Ph 2,6-H), 8.57 (1H, s, 3-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 22.8 (Me), 55.66 (4-MeO), 55.69 (2-MeO), 98.9 (NH-Ph C-3), 103.4 (NH-Ph C-5), 116.1 (NH-Ph C-6), 121.1 (C-3a), 121.2 (N-Ph C-2,6), 121.6 (C-3), 123.4 (C-7), 127.6 (C-Ph C-4), 127.8 (NH-Ph C-1), 128.4 (C-Ph C-3,5), 128.5 (N-Ph C-4), 129.0 (C-Ph C-2.6), 129.7 (N-Ph C-3.5), 139.4 (C-Ph C-1), 140.2 (N-Ph C-1), 140.9 (C-6), 148.1

(C-7a), 149.4 (C-4), 149.6 (NH-Ph C-2), 154.0 (NH-Ph C-4). ^{15}N NMR (51 MHz, $CDCl_3$): δ −316.2 (NH), −149.2 (N-2), −98.8 (N-5), −83.4 (N-1). IR (ν, cm^{-1}): 3382 (NH), 3053, 2997 (CH_{arom}), 2933, 2832 (CH_{aliph}), 1595, 1511, 1463, 1283, 1204 (C=C, C=N, C–N), 1155, 1029 (C-O-C), 829, 756, 696, 689 (CH=CH of mono- and trisubstituted benzenes). MS (ES^+): *m/z* (%): 437 ($[M+H]^+$, 98.7). HRMS (ESI) $C_{27}H_{25}N_4O_2$ ($[M+H]^+$) calcd 437.1972, found 437.1972.

4.1.2.1.14 *N*-(3,4,5-Trimethoxyphenyl)-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 17

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 3,4,5-trimethoxyaniline (24 mg, 0.13 mmol), NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 36 mg (63%), reddish brown crystals, mp = 107–108 °C, R_f = 0.31 (Hex:EtOAc 1:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.93 (3H, s, CH_3), 3.66 (6H, s, 3,5-OCH_3), 3.75 (3H, s, 4-OCH_3), 6.06 (2H, s, NH-Ph 2,6-H), 6.07-6.09 (1H, br s, NH), 7.26-7.30 (1H, m, C-Ph 4-H), 7.34-7.38 (2H, m, C-Ph 3,5-H), 7.43-7.47 (1H, m, N-Ph 4-H), 7.21-7.56 (2H, m, N-Ph 3,5-H), 7.73-7.77 (2H, m, C-Ph 2,6-H), 7.87-7.90 (2H, m, N-Ph 2,6-H), 8.62 (1H, s, 3-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 22.8 (CH_3), 56.0 (3,5-OCH_3), 61.1 (4-OCH_3), 95.0 (NH-Ph C-2,6), 121.1 (C-3a), 121.2 (N-Ph C-2,6), 122.0 (C-7), 122.6 (C-3), 127.8 (C-Ph C-4), 128.5 (C-Ph C-3.5), 128.7 (N-Ph C-4), 129.0 (C-Ph C-2.6), 129.8 (N-Ph C-3.5), 132.1 (NH-Ph C-4), 139.4 (NH-Ph C-1), 140.0, 140.3 and 140.5 (C-6, C-Ph C-1 or N-Ph C-1), 148.0 (C-7a), 150.0 (C-4), 153.3 (NH-Ph C-4,5). IR (ν, cm^{-1}): 3346, 3314 (NH), 3112, 3057 (CH_{arom}), 2923, 2852 (CH_{aliph}), 1596, 1502, 1463, 1412, 1232 (C=C, C=N, C–N), 1125, 1006 (C-O-C), 804, 757, 690 (CH=CH of mono- and tetrasubstituted benzenes). MS (ES^+): *m/z* (%): 467 ($[M+H]^+$, 100). HRMS (ESI) $C_{28}H_{27}N_4O_3$ ($[M+H]^+$) calcd 467.2078, found 467.2078.

4.1.2.1.15 4-Methyl-2,6-diphenyl-*N*-[2-(trifluoromethoxy)phenyl]-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 18

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 2-(trifluoromethoxy)aniline (23 mg, 0.13 mmol),

NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 46 mg (82%), yellowish crystals, mp = 113–114 °C, R_f = 0.62 (Hex:EtOAc 1:1).^{1}H NMR (500 MHz, $CDCl_3$): δ 2.87 (3H, s, CH_3), 6.28 (1H, s, NH), 6.68-6.77 (2H, m, NH-Ph 4,6-H), 6.87-6.93 (1H, m, NH-Ph 3-H or 5-H), 7.16-7.21 (1H, m, NH-P, 3-H or 5-H), 7.24-7.29 (1H, m, C-P, 4-H), 7.32-7.36 (2H, m, C-Ph 3,5-H), 7.40-7.44 (1H, m, N-Ph 4-H), 7.49-7.53 (2H, m, N-Ph 3,5-H), 7.73-7.79 (2H, m, C-Ph 2,6-H), 7.85-7.89 (2H, m, N-Ph 2,6-H), 8.56 (1H, s, 3-H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 23.0 (CH_3) 116.7 (NH-Ph C-6), 117.9, 119.2, 124.0 (CF_3 J =252 Hz), 120.7 (NH-Ph C-3 or C-5), 120.8 (C-7), 121.1 (N-Ph C-2,6), 121.2 (C-3a), 122.0 (C-3), 126.7 (NH-Ph C-3 or C-5), 127.9 (C-Ph C-4), 128.5 (C-Ph C-3,5), 128.7 (N-Ph C-4), 128.8 (C-Ph C-2,6), 129.7 (N-Ph C-3,5), 136.8 (NH-Ph C-1), 137.7 (NH-Ph C-2), 139.1 (C-Ph C-1), 140.0 (N-Ph C-1), 143.1 (C-6), 148.3 (C-7a), 151.5 (C-4). IR (ν, cm^{-1}): 3412, 3396 (NH), 3133, 3056 (CH_{arom}), 2920, 2852 (CH_{aliph}), 1600, 1511, 1485, 1434, 1248, 1213, 1167 (C=C, C=N, C–N, C-F), 755, 697, 688 (CH=CH of mono- and disubstituted benzenes). MS (ES^+): *m/z* (%): 461 ($[M+H]^+$, 100). HRMS (ESI) $C_{26}H_{20}F_3N_4O$ ($[M+H]^+$) calcd 461.1584, found 461.1584.

4.1.2.1.16 4-Methyl-2,6-diphenyl-*N*-[3-(trifluoromethoxy)phenyl]-2*H*-pyrazolo[4,3-*c*]pyridin-7-amine 19

Prepared in accordance to general procedure from 7-iodo-4-methyl-2,6-diphenyl-2*H*-pyrazolo[4,3-*c*]pyridine **3** (50 mg, 0.12 mmol), 3-(trifluoromethoxy)aniline (23 mg, 0.13 mmol), NaO*t*Bu (18.7 mg, 0.19 mmol), SPhos (15 mg, 0.036 mmol) and $Pd(OAc)_2$ (1.7 mg, 0.012 mmol) and dioxane (4 mL). The residue was purified by column chromatography (EtOAc/PE, 1:4 to 1:2, v/v).

Yield: 35mg (62%), yellowish crystals, mp = 76–78 °C, R_f = 0.59 (Hex:EtOAc 1:1). ^{1}H NMR (700 MHz, $CDCl_3$): δ 2.87 (3H, s, CH_3), 5.99 (1H, s, NH), 6.63-6.65 (1H, m, NH-Ph 2-H), 6.65-6.67 (1H, m, NH-Ph 4-H), 6.68-6.72 (1H, m, NH-Ph 6-H), 7.10-7.14 (1H, m, NH-Ph 5-H), 7.28-7.31 (1H, m, C-Ph 4-H,), 7.36-7.39 (2H, m, C-Ph 3,5-H), 7.42-7.45 (1H, m, N-Ph 4-H), 7.50-7.53 (2H, m, N-Ph 3,5-H), 7.69-7.72 (2H, m, C-Ph 2,6-H), 7.84-7.88 (2H, m, N-Ph 2,6-H), 8.57 (1H, s, 3-H). ^{13}C NMR (176 MHz, $CDCl_3$): δ 22.9 (CH_3), 108.8 (NH-Ph C-2), 111.7 (NH-Ph C-4), 114.7 (NH-Ph C-6), 119.7 (OCF_3), 121.0 (C-7), 121.1 (C-3a), 121.2 (N-Ph C-2,6), 122.0 (C-3), 127.8

(C-Ph C-4), 128.5 (C-Ph C-3,5), 128.6 (N-Ph C-4), 128.9 (C-Ph C-2,6), 129.5 (NH-Ph C-5), 129.6 (N-Ph C-3,5), 138.8 (C-Ph C-1), 139.9 (N-Ph C-1), 142.9 (C-6), 146.1 (NH-Ph C-1 or C-3), 147.9 (C-7a), 149.8 (NH-Ph C-1 or C-3), 151.4 (C-4). ^{15}N NMR (71 MHz, $CDCl_3$): δ −304.5 (NH), −148.3 (N-2), −82.2 (N-5). IR (ν, cm^{-1}): 3384 (NH), 3059 (CH_{arom}), 2923, 2853 (CH_{aliph}), 1610, 1598, 1519, 1487, 1249, 1213, 1178, 1151 (C=C, C=N, C–N, C-F), 756, 697, 689 (CH=CH of mono- and disubstituted benzenes). MS (ES^+): *m/z* (%): 461 ($[M+H]^+$, 100). HRMS (ESI) $C_{26}H_{20}F_3N_4O$ ($[M+H]^+$) calcd 461.1584, found 461.1583.

4.2. Optical measurements

Stock solutions (4 mM) of the compounds were prepared in DMSO, which were further diluted to a final concentration of 4 μM in pH 7 Britton–Robinson buffer, a solution consisting of 0.04 M H_3PO_4, 0.04 M CH_3COOH and 0.04 M H_3BO_3, which was adjusted to pH 7 by 0.2 M NaOH. Absorption spectra were measured using a Specord 250 Plus spectrophotometer in a 250-500 nm range with the step of 1 nm, 1 nm bandpass and 2 nm/s scan-speed. Fluorescence emission spectra were measured in a 400-700 nm range with excitation at 380 nm using Fluorolog-3 (Horiba) spectrofluorometer. Bandpasses in both the excitation and emission were set to 5 nm, the spectra were scanned with the 1 nm step and integration time 0.2 s per data point. Signal of pure solvent was subtracted as a background.

4.3. Biology

4.3.1. Cell cultures

G361 cell line (human skin melanoma) was cultivated in DMEM medium and HaCaT cell line (immortalized keratinocytes) was cultivated in DMEM with high glucose (4.5 g/L) medium. Medium was supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) and cells were cultivated at 37 °C in 5% CO_2 atmosphere.

4.3.2. Photodynamic treatment

An in-house constructed LED based light source specifically designed for the irradiation of 96-well microplates and Petri dishes [56] was used; a maximal wavelength emission of 414 nm, light intensity set to 20 mW/cm^2. The total dose of irradiation used was 1, 5 or 10 J/cm^2 and there were no significant changes of temperature during irradiation. For photodynamic treatments, cells were

seeded and next day treated with test compounds; after 4 h incubation, cells were irradiated and cultivated for further 24 h or 72 h.

4.3.3. Cell viability assay

Cell viability was determined using the MTT (Sigma-Aldrich) assay in 96-well microplates (5000 cells per well). The test compounds were added 24 h post plating, the cells were then incubated for additional 4 h and irradiated (the maximal wavelength emission of 414 nm and total dose of 1, 5 or 10 J/cm^2). After irradiation, the cells were incubated for further 72 h and then the MTT solution was added, cells were incubated for another 4 h at 37 °C and then the 0.1% SDS was added to the wells to solubilize the violet formazan crystals. The measurement of absorbance was carried out on reader Tecan Infinite M200Pro at 570 nm. Dark viability was measured in parallel under the same conditions without irradiation.

4.3.4. Measurement of reactive oxygen species (ROS) production

The cells were treated for 4 h with compound **10** and then ROS was quantified using a CM-H_2DCFDA (Invitrogen), which is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. After adding 50 µl of H_2DCFDA solution (10 µl 10 µM DCF + 1 ml PBS).and 25 min incubation, the cells were irradiated and the ROS production was determined by measuring fluorescence using Tecan Infinite M200Pro at 480/540 (excitation/emission) as described earlier [42].

4.3.5. Flow cytometry

Asynchronous cells were seeded and, after a preincubation period, treated with test compound for 4 h, irradiated and after further 24h of incubation, samples were fixed and stained with propidium iodide. DNA content was analyzed by flow cytometry using a 488 nm laser (BD FACS Verse with software BD FACSuite^{TM}, version 1.0.6.) and cell cycle distribution was analyzed using ModFit LT (Verity Software House).

4.3.6. Caspase-3/7 assay

The cell lysates were incubated for 4 h with 100 μM Ac-DEVD-AMC (the substrate of caspases 3 and 7) in the assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems) at 355/460 nm (excitation/emission).

4.3.7. Comet assay

Treated G361 cells were trypsinized, rinsed by DMEM and then the cell suspension in 1% LMP agarose was pipetted to the microscope slides with agarose gel. The microscope slides were immersed in a lysis buffer with 1% Triton X for 1 h and then placed in an electrophoretic tank and dipped into a cool electrophoresis solution for 40 min. After the electrophoresis (20 V, 350 mA, 20 min), the microscopic slides were immersed in a neutralisation buffer (10 min twice). The samples were then stained by SYBR Green (Invitrogen) for 15 min and scored by the SW Comet Score (TriTek Corp.).

4.3.8. Immunoblotting

Cell lysates were prepared in RIPA buffer. Equal protein amounts (17 μg/well) were loaded and separated on SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes and after blocking, overnight incubation with specific primary antibodies and incubation with peroxidase-conjugated secondary antibodies, the peroxidase activity was detected with SuperSignal West Pico reagents (Thermo Scientific) using a CCD camera LAS-4000 (Fujifilm). All primary antibodies were diluted in TBS containing 4% BSA and 0.1% Tween 20. The specific antibodies were purchased from Cell Signaling (anti-PARP, clone 46D11; anti-cleaved caspase 7; anti-Mcl-1, clone D35A5; HRP-linked secondary antibodies), Sigma Aldrich (anti-α-tubulin, clone DM1A), Millipore (anti-phospho-histone H2A.X, Ser139, clone JBW301).

CRediT authorship contribution statement

Beatričė Razmienė: Investigation, Formal analysis, Writing - Original Draft. **Veronika Vojáčková**: Investigation, Formal analysis. **Eva Řezníčková**: Investigation, Formal analysis, Visualization. **Lukáš Malina**: Investigation, Formal analysis. **Vaida Dambrauskienė**: Investigation. **Martin Kubala**: Investigation, Formal analysis. **Robert Bajgar**: Resources, Methodology. **Hana Kolářová**: Supervision, Resources, Funding acquisition. **Asta Žukauskaitė**:

Supervision, Visualization, Writing - Original Draft, Writing - Review & Editing. **Eglė Arbačiauskienė**: Methodology, Supervision, Funding acquisition, Writing - Original Draft, Writing - Review & Editing. **Algirdas Šačkus**: Conceptualization, Resources. **Vladimír Kryštof**: Conceptualization, Supervision, Resources, Writing - Original Draft, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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