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Discovery and selectivity-profiling of 4-benzylamino 1-aza-9-oxafluorene derivatives as lead structures for IGF-1R inhibitors

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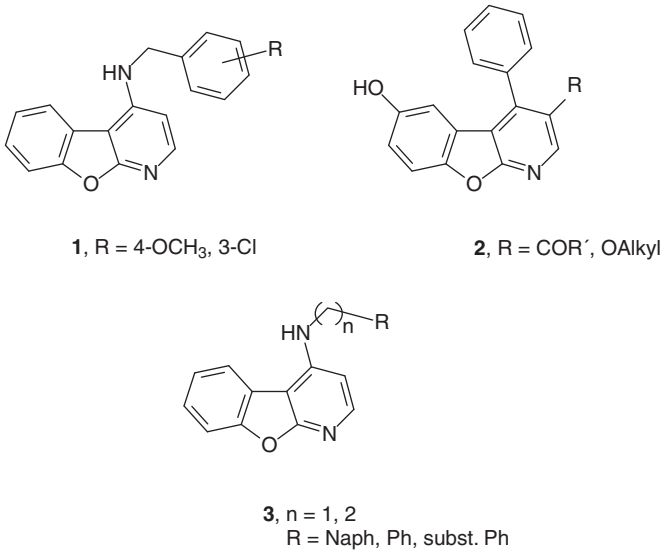
ABSTRACT

Recently the insuline-like growth factor receptor (IGF-1R) emerged as a promising target structure for the development of novel anti-cancer agents. IGF-1R plays a central role in both tumour progression and resistance development against anti-cancer drugs. We discovered 1-aza-9-oxafluorene derivatives as novel lead structures with submicromolar activities against IGF-1R. Structure–activity relationships (SARs) on a series of related receptor tyrosine kinases (RTKs) are discussed in the context of available crystal structures. A preliminary selectivity-profiling is demonstrated for the first compound series. Antiproliferative tumour cell line screening studies yielded one candidate as a promising cytostatic agent without significant toxic effects.

The resistance development in cancer treatment emerged to a central problem in the therapy with established cytostatics.[1–3] Also novel anti-cancer compounds like tyrosine kinase (TK) inhibitors are affected by the resistance phenomenon. Lowered sensitivities which are partly caused by mutations of amino acids in the ATP binding pocket are reported in the case of clinically used inhibitors of the epidermal growth factor receptor (EGFR).[4,5] Another important feature in the resistance development of TK inhibitors is the heterodimerization of EGFR with the kinase insuline-like growth factor receptor (IGF-1R).[6–9] This kinase is overexpressed in many cancer tissues.[10,11] The overexpression leads to an enhanced cell proliferation and tumour growth. Additionally, inhibition of cancer cell apoptosis is influenced by IGF-1R, so that resistance development against radiation therapy is also enforced by this kinase.[10]

IRF-1R emerged as a promising target for the therapy of cancer and cancer resistance. We discovered 4-benzylamino 1-aza-9-oxafluorene **1** as novel lead structure for IGF-1R inhibitors (Fig. 1). Structure–activity relationships (SARs) on related receptor tyrosine kinases (RTKs) are discussed based on enzyme inhibition data as well as on available kinase crystal structures. Docking studies using the EGFR, IGF-1R and VEGFR2 crystal structures were carried out to rationalise the obtained biological data. First selectivity-profiling data and antiproliferative activities are reported.



1, R = 4-OCH_3, 3-Cl

2, R = COR´, OAlkyl

3, n = 1, 2
R = Naph, Ph, subst. Ph

Figure 1. 1-Aza-9-oxafluorene lead structure **1**, cycline dependent kinase inhibiting 1-aza-9-oxafluorene **2** and substituted 4-arylalkylamino template structure **3** of the presented synthesised series.

First 1-aza-9-oxafluorenes **2** having a substituent in the 3-position of the aromatic scaffold and a 6-hydroxy function were reported to show cycline dependent kinase (CDK) inhibiting

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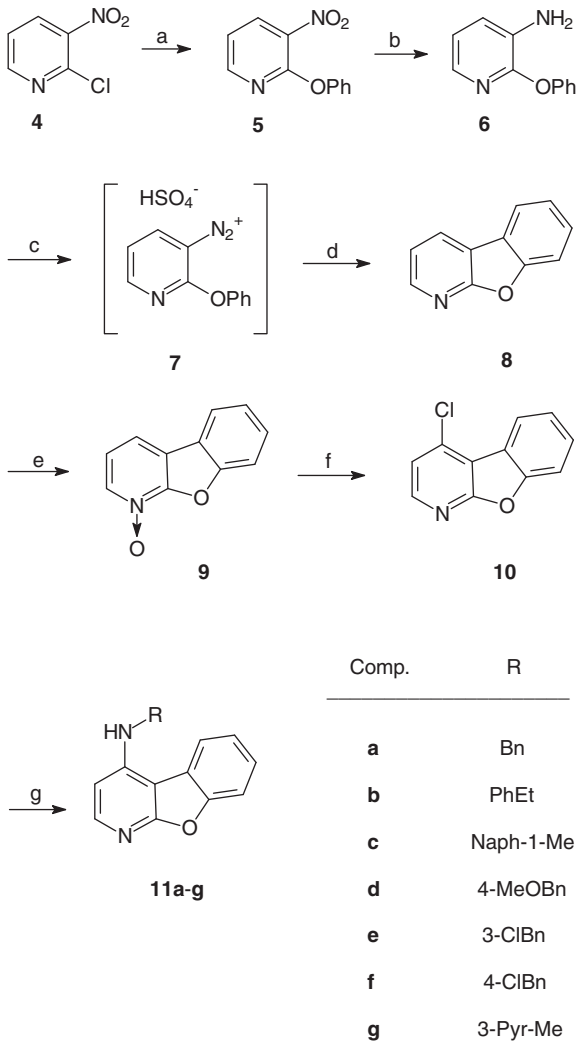
activity depending on the nature of the 3-substituent.[12,13] An acylation of the 6-hydroxy function led to a loss of activity.[12] The presence of a 4-aryl substituent was found to be important for CDK activity.[12]

We further varied the substitution pattern of the promising 1-aza-9-oxafluorene scaffold. First, we eliminated the 6-hydroxy function to reduce the CDK inhibiting activity. Additionally, the 3-substituent was removed. Instead of the 4-aryl substituent we introduced a 4-arylalkylamino function into the template structure **3**. In this way the important aryl function in the 4-position was maintained. In addition, the aryl function was enlarged by an amino alkylene group. The amino function was introduced as a potential hydrogen bond donor or acceptor function, respectively. The synthesis of the 1-aza-9-oxafluorene scaffold started from 2-chloro-3-nitropyridine **4** which was treated with phenolate anion (generated from phenole under proton abstraction with sodium hydride) (Scheme 1).[14]

The resulting 2-phenoxy-3-nitropyridine **5** was reduced to the amino derivative **6** using hydrogen and palladium on charcoal as catalyst.[14] The cyclisation reaction of **6** to the 1-aza-9-oxafluorene **8**[15] started with the formation of a diazonium salt intermediate **7** using sodium nitrite in sulphuric acid and a consequent ring closure using copper as catalyst. The *N*-oxide **9**[16] was given by an oxidation of compound **8** with *meta* chloroperbenzoic acid. The reaction of the *N*-oxide **9** with phosphoroxychloride led to the 4-chloro-1-aza-9-oxafluorene **10**[17] as template for the following amine derivatization reactions. The different arylalkylamino compounds **11a**–**g** were given by boiling a mixture of the respective amine and the 4-chloro-1-aza-9-oxafluorene **10**.[18] All novel compounds were characterised by IR, ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry and elemental analysis.



Scheme 1. Reagents and conditions for compounds **4**–**11a**–**g**: (a) PhOH, NaH, toluene, reflux, 3.5 h (100%); (b) H_2/Pd/C, ethyl acetate, rt (98%); (c) $NaNO_2$, H_2SO_4, 0 °C; (d) Cu, H_2SO_4, 0 °C up to 55 °C, 90 min (31%); (e) *m*-chloroperbenzoic acid, $CHCl_3$, reflux, 8 h (100%); (f) phosphoroxychloride, $CHCl_3$, 100 °C, 3.5 h (79%); (g) arylalkylamine, 135–145 °C (51%—**a**, 62%—**b**, 60%—**c**, 61%—**d**, 42%—**e**, 51%—**f**, 65%—**g**).

First screening results derived from a panel of RTKs showed that some of the compounds show a preference for IGF-1R, EGFR and VEGFR2. For example, the 4-benzylamino derivative **11a** showed submicromolar activity in the inhibition of IGF-1R, EGFR and of the vascular endothelian growth factor 2 (VEGFR2) (Table 1).

The simultaneous inhibition of these kinases is attractive for the treatment of cancer with overexpressing EGFR and VEGFR2 in the late state of metastasis because both kinases are involved in enhanced cell viability, cell adhesion and invasion processes beside their role in angiogenesis.[19–24] An elongation of the alkyl side chain by just one methylene function (compound **11b**) led to a decrease in activity of the RTK inhibition. Since the size of a hydrophobic pocket near the gatekeeper residue, which is predicted by the docking studies to be the binding site of the 4-aminobenzyl group, is restricted in several kinases, the elongation of this group is unfavourable.[25] The naphthyl substituent in compound **11c** is more bulky than the phenyl substituent in compound **11a** but was found to adopt a similar location in the binding pocket as observed for the phenyl ring. The activity data of compound **11c** are similar to those of the benzylamine derivative **11a**. An interesting change in activity was observed for the 4-methoxyphenyl derivative **11d**. Good activities for the inhibition of IGF-1R and EGFR were found for compound **11d** while no inhibition of both VEGFR2 and VEGFR3 was observed. Derivative **11d** represents a dual inhibitor of both IGF-1R and EGFR and thus is indicated as a novel RTK inhibitor in EGFR overexpressing cells. The combination of IGF-1R with EGFR as addressed inhibitor target structures will mainly lower a resistance development against the EGFR inhibition ability by preventing the effect of a heterodimerization because an IGF-1R/EGFR heterodimer will also be inhibited by the dual inhibitor **11d**.[6,7,9]

The 3-chloro derivative **11e** exclusively inhibited IGF-1R and thus is indicated in combination with established EGFR inhibitors to lower the occurring resistance effect caused by heterodimerization of both kinases. The 4-chloro derivative **11f** was found as a multikinase inhibitor with highest affinity for the investigated RTKs within this first series. The inhibition of VEGFR3 is of worth also because of the role of VEGFR3 in lymphatic angiogenesis.[26] Finally, a pyridine ring was used as terminal aromatic system of the 4-benzylamino group. No activity was found for the resulting 3-pyridyl derivative **11g**.

Table 1
Biological activity data for the 4-arylalkylamino substituted target compounds **11a**–**g**

Compound	K_i values[a,b] (μM)			
	IGF-1R	EGFR	VEGFR2	VEGFR3
11a	0.37	0.59	0.47	1.09
11b	2.11	3.45	2.01	9.39
11c	0.57	0.68	0.30	1.09
11d	0.15	0.40	na[c]	na[c]
11e	0.58	na[c]	na[c]	na[c]
11f	0.11	0.50	0.18	0.68
11g	na[c]	na[c]	na[c]	na[c]

[a] K_i values have been calculated from determined IC_{50} values of RTK inhibition following described protocols.[12,13]
[b] Standard errors are typically below 20%. In many cases standard errors below 10% are found.
[c] Inactive, K_i >1000 μM.

In order to provide a potential rationalisation for the detected in vitro activities docking studies were carried out using the available crystal structures of human IGF-1R, EGFR and VEGFR2 in the active form (PDB code 2zm3, 2ity, and 2p2h, respectively). All three kinases have been crystallized with inhibitors addressing the active form of the kinases. The inhibitors were docked into the ATP binding pocket using the program GOLD (Cambridge Crystallographic Data Centre, Cambridge, UK) and default docking settings. When docked to the three kinases, the 1-aza-9-oxafluorene ring system is mimicking the adenine ring of ATP and represents a hinge-binding motif. The inhibitors show a hydrogen bond between the pyridine nitrogen and the backbone NH of the hinge residue Met793 (EGFR), Met1082 (IGF-1R) and Cys919 (VEGFR2), respectively (Fig. 2a–c). The 4-benzylamino substituent is located in a hydrophobic pocket nearby the gatekeeper residue (Thr790 in EGFR, Met1079 in IGF-1R and Thr916 in VEGFR2). The varying gatekeeper residue and the size of the pocket are mainly responsible for the observed selectivity of some inhibitors. In general, hydrophobic substituents are favoured in this pocket and polar groups (as in **11g**) tend to stick out of this pocket resulting in inactive derivatives. IGF-1R contains the more hydrophobic methionine as gatekeeper residue (threonine in EGFR and VEGFR2) resulting in higher affinities of the inhibitors possessing hydrophobic substituents at the 4-benzylamino group (**11e, 11f**).

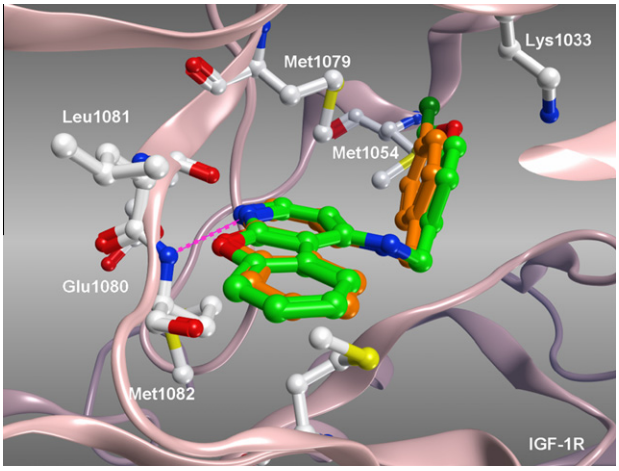


Figure 2a. GOLD docking results for IGF-1R. The two potent inhibitors **11d** (coloured green) and **11f** (coloured orange) show favourable interaction between the substituted 4-benzylamino group and the hydrophobic pocket nearby the gatekeeper residue (Met1079). The hydrogen bond to the hinge region (Met1082) is shown as dashed line.

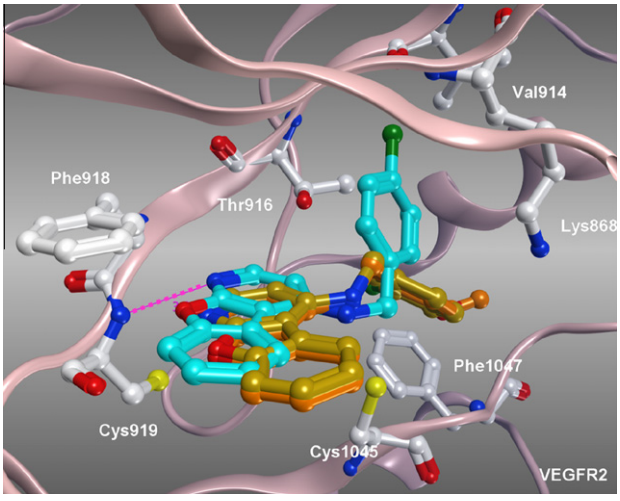


Figure 2b. GOLD docking results for VEGFR2. The potent inhibitor **11f** (coloured cyan) interacts with the hydrophobic pocket nearby the gatekeeper residue (Thr916). For the two inactive derivatives **11d** (coloured orange) and **11e** (coloured dark yellow) the docking shows that the substituted 4-benzylamino group cannot be accomodated in this pocket. As a result the important hydrogen bond to the hinge residue (Cys919) is lost.

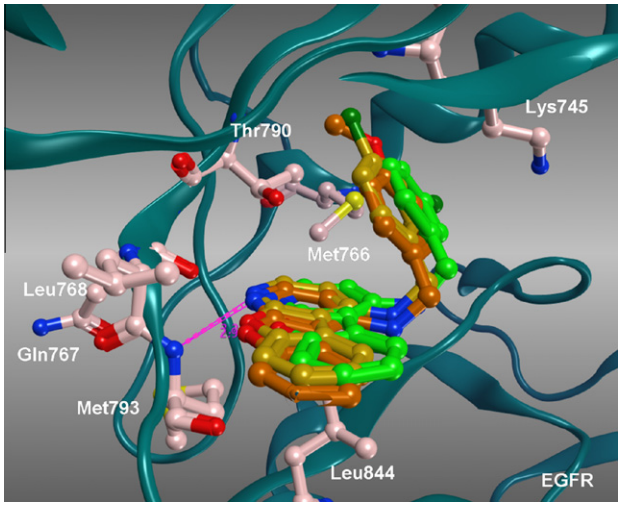


Figure 2c. GOLD docking results for EGFR. The docking study shows that in case of the most active inhibitors **11d** (coloured orange) and **11f** (coloured dark yellow) the 4-benzylamino group is interacting with the gatekeeper pocket whereas for **11e** (coloured green) the substituted aromatic ring is pushed away from Met766.

Due to the larger hydrophobic pocket in IGF-1R (Fig. 2a) this kinase accepts all 1-aza-9-oxafluorene derivatives, whereas in the EGFR and VEGFR2 crystal structure the corresponding pocket is smaller. As a result, the docking of **11d** and **11e** in VEGFR2 showed, that the substituted 4-benzylamino ring cannot be fully buried in this hydrophobic pocket (Fig. 2b) resulting in a loss of the hydrogen bond between pyridine nitrogen and the hinge region (Cys919). A similar effect was observed for **11e** at EGFR (Fig. 2c). Also here, the size of the pocket nearby the gatekeeper is responsible for a slightly different orientation of **11e** compared to the other inhibitors. As a consequence the distance of the important hydrogen bond to the hinge region (Met793) is increased. In general, a good agreement between the docking results and the biological activities at IGF-1R, EGFR and VEGFR2 was observed.

The most potent IGF-1R/EGFR inhibitor **11d** has been preliminarily screened against 27 kinases from almost all families of the human kinome. This was done to widely estimate the potential of being well-tolerated in an anti-cancer therapy because of a selective protein kinase inhibiting activity. To the best of our knowledge, no selective IGF-1R inhibitor has been reported so far.[10,27,28]

The biological activities from the kinase panel are shown in Table 2. All determined activity data range from 100 μM to 1000 μM. This is a concentration range of practically no activity. Complete loss of activity was found in the inhibition of 18 kinases. It is noteworthy that no inhibitory activity was observed for PDGFR-β and TIE2 because both are related kinases from the RTK family. More interestingly we found an impressing selectivity in the IGF-1R inhibition compared to the inhibition of the closely related RTKs of both the InsR (insuline receptor) and the InsRR (insuline receptor receptor) kinases which were practically not affected by our inhibitor. So we found a first highly selective protein kinase inhibitor as far as evaluated.

The National Cancer Institute of Health (NCI) selected the multikinase inhibitor **11f** for a tumour cell line screening. The antipro-

Table 2
Selectivity profile from the screening of protein kinase inhibition for compound **11d**

Kinase family[a]	Kinase	IC_{50} value[b] (μM)
Tyrosine kinase (TK) family	ABL1	392
	ABL2	na[c]
	FAK	194
Receptor tyrosine kinase (RTK) family	InsR	622
	InsRR	182
	PDGFR-β	na[c]
	TIE2	na[c]
Tyrosine kinase like (TKl) family	IRAK1	na[c]
	LIMK1	759
Cyclin dependent kinase (CDK) family	CDK4/D1	376
	CDK6/D1	129
	CDK5/p25	na[c]
CDK-like family	CLK1	na[c]
	DYRK1A	na[c]
	HIPK1	na[c]
Protein kinase A (PKA) family	Aurora A	na[c]
	Auroara C	na[c]
	PKC-γ	na[c]
	PKC-iota	na[c]
Mitogen activated protein kinase (MAPK) family	ERK1	na[c]
	p38-α	na[c]
	INK1	na[c]
Casein kinase (CK) family	CK1-α1	na[c]
	VRK1	129
	WEE1	na[c]
Nek protein kinase family	Nek3	na[c]
Polo subfamily	PLK1	271

[a] Kinase families as reported in Ref. 32.
[b] IC_{50} value determination followed recent protocols.[12,13]
[c] Inactive, K_i >1000 μM.

Table 3
Anti-cancer screening data of 60 tumour cell lines as mean graph midpoint (MG_MID) values for inhibitor **11f** in comparison to used cytostatics

Compound	MG_MID values	
	log GI_{50}	log LC_{50}
11f	−4.47	>−4.00
Etoposide	−4.41	>−4.00
Melphalan	−4.48	−4.04
Irinotecan	−4.85	−4.02
Cisplatin	−5.68	−5.60

liferative activities were characterised as mean graph midpoint values for a reduced cell proliferation shown as log GI_{50} value and for the lethal toxicity as log LC_{50} value determined in a sulphur rhodamine B cytotoxicity assay.[29,30]

The results are shown in Table 3 in comparison to NCI database values for established cytostatic drugs used in anti-cancer therapies.[31]

The antiproliferative activities as log GI_{50} values prove our inhibitor **11f** as active as etoposide, melphalan or irinotecan. Furthermore, our compound showed the lowest toxicity in among melphalan, irinotecan or cisplatin. Their higher toxicities are known to be critical because such a general toxicity causes severe side effects in anti-cancer therapy.

In the current work we discovered a new class of selective IGF-1R inhibitors which are qualified for a cancer resistance therapy as dual IGF-1R/EGFR inhibitors and as exclusive IGF-1R inhibitors for an anti-cancer therapy with EGFR-resistant inhibitors. They are the first reported small molecule IGF-1R inhibitors with a proved and characterised selectivity profile. Moreover, they are suggested as well-tolerated anti-cancer drugs showing no general toxicity and an antiproliferative potential similar to clinically used drugs. Further derivatizations guided by the docking results are ongoing to increase the affinity of the inhibitors.

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15. Spectroscopical data of 1-aza-9-oxafluorene **8**: mp 57–61 °C; ^{1}H NMR ($CDCl_3$) δ = 7.31 (dd, *J* = 7.7, 4.9 Hz, 1H), 7.36 (ddd, *J* = 8.3, 7.6, 0.9 Hz, 1H), 7.50 (ddd, *J* = 8.3, 7.3, 1.7 Hz, 1H), 7.62 (d, *J* = 7.3 Hz, 1H), 7.92 (dd, *J* = 7.7, 0.5 Hz, 1H), 8.26 (dd, *J* = 7.6, 1.7 Hz, 1H), 8.43 (d, *J* = 4.9 Hz, 1H); *m*/*z* (ESI) 170 ($M+H^+$).
16. Spectroscopical data of 1-aza-9-oxafluorene-*N*-oxide **9**: mp 176–178 °C; ^{1}H NMR ($CDCl_3$) δ = 7.28 (dd, *J* = 7.7, 6.6 Hz, 1H), 7.45 (ddd, *J* = 8.2, 7.3, 0.8 Hz, 1H), 7.59 (ddd, *J* = 8.3, 7.3, 1.2 Hz, 1H), 7.71 (d, *J* = 8.3 Hz, 1H), 7.82 (d, *J* = 7.7 Hz, 1H), 7.95 (dd, *J* = 8.2 Hz, 1H), 8.32 (d, *J* = 6.6 Hz, 1H); *m*/*z* (ESI) 186 ($M+H^+$).
17. Spectroscopical data of 4-chloro-1-aza-9-oxafluorene **10**: mp 78–79 °C; ^{1}H NMR ($CDCl_3$) δ = 7.32 (d, *J* = 5.4, 1H), 7.45 (ddd, *J* = 8.3, 7.4, 0.9 Hz, 1H), 7.59 (ddd, *J* = 8.3, 7.4, 1.3 Hz, 1H), 7.64 (d, *J* = 8.3 Hz, 1H), 8.25 (d, *J* = 8.3 Hz, 1H), 8.32 (d, *J* = 5.4 Hz, 1H); *m*/*z* (ESI) 204 ($M+H^+$).
18. General procedure for the formation of the arylalkylamino target compounds **11a**–**g**: compound **10** (0.25 g, 1 mmol) was dissolved in the respective amine (25 mmol) and the mixture was heated at 135 °C for 20 h under argon atmosphere. Then the mixture was poured into a saturated potassium carbonate solution (25 mL) and ethyl acetate (50 mL) was added. After the phase separation the water phase was extracted with ethyl acetate (25 mL) for three times. The unified organic layers were dried over sodium sulphate, filtered and the eluent was removed in vacuum. The resulting products were each given by column chromatography over silica gel using mixtures of cyclohexane and ethyl acetate. Characterising spectroscopical data of representing selective derivatives: compound **11d**: mp 148–154 °C; IR (KBr) ν = 3319, 3058, 2995, 1604, 1584; ^{1}H NMR (DMSO-d_6) δ = 3.09 (s, 3H), 4.56 (d, *J* = 6.0 Hz, 2H), 6.49 (d, *J* = 5.9 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.37–7.48 (m, 3H), 7.63 (d, *J* = 7.9 Hz, 1H), 7.91 (d, *J* = 5.9 Hz, 1H), 8.43 (d, *J* = 7.5 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ = 45.8, 55.6, 100.6, 102.5, 111.4, 114.4 (2 × C), 122.0, 122.8, 123.4, 126.3, 128.5 (2 × C), 131.3, 147.4, 150.5, 152.2, 158.7, 164.4; *m*/*z* (EI) 304 (M^+). Elemental Anal. Calcd (%) for $C_{19}H_{16}N_2O_2$: C, 74.98; H, 5.30; N, 9.20. Found: C, 74.65; H, 5.41; N, 8.83. Compound **11e**: mp 240–243 °C; IR (KBr) ν = 3308, 3059, 2868, 1604, 1583; ^{1}H NMR (DMSO-d_6) δ = 4.65 (d, *J* = 6.1 Hz, 2H), 6.49 (d, *J* = 5.9 Hz, 1H), 7.26–7.31 (m, 1H), 7.32–7.53 (m, 6H), 7.65 (d, *J* = 7.8 Hz, 1H), 7.93 (d, *J* = 5.9 Hz, 1H), 8.44 (d, *J* = 7.1 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ = 45.8, 100.8, 102.4, 111.4, 122.1, 122.7, 123.4, 126.0, 126.5, 127.1, 127.3, 130.8, 133.6, 142.3, 147.5, 150.3, 152.3, 164.4; *m*/*z* (EI) 308 (M^+); Elemental Analy. Calcd (%) for $C_{18}H_{13}ClN_2O$: C, 70.02; H, 4.24; Cl, 11.48; N, 9.07. Found: C, 70.11; H, 4.01; Cl, 11.53; N, 8.87.
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