

# Synthesis and antiproliferative activity of 2-([1,2,4]triazolo[4,3-*b*]-pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine derivatives

Miloš Ilić,<sup>a</sup> Janez Ilaš,<sup>a</sup> Sandra Liekens,<sup>b</sup> Péter Mátyus,<sup>c</sup> and Danijel Kikelj<sup>a,\*</sup>

<sup>a</sup>University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia

<sup>b</sup>Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium

<sup>c</sup>Semmelweis University, Department of Organic Chemistry, Hőgyes E. u. 7, 1092 Budapest, Hungary

E-mail: [danijel.kikelj@ffa.uni-lj.si](mailto:danijel.kikelj@ffa.uni-lj.si)

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## Abstract

A small library of [1,2,4]triazolo[4,3-*b*]pyridazin-6-yloxy derivatives **14-17** of *N*-benzyl-*N*-(2-((4-amidinophenoxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazin-7-yl)-oxalic acid monoamides **1** was prepared by replacement of benzamidine with a [1,2,4]triazolo[4,3-*b*]pyridazine-6-yl moiety. Thrombin inhibitory and fibrinogen receptor antagonistic activities inherent to benzamidine compounds **1** were lost in the [1,2,4]triazolo[4,3-*b*]pyridazine derivatives **14-17** which, on the other side, in their ester form ( $R^2 = Et$ ) inhibited the proliferation of endothelial and tumor cells.

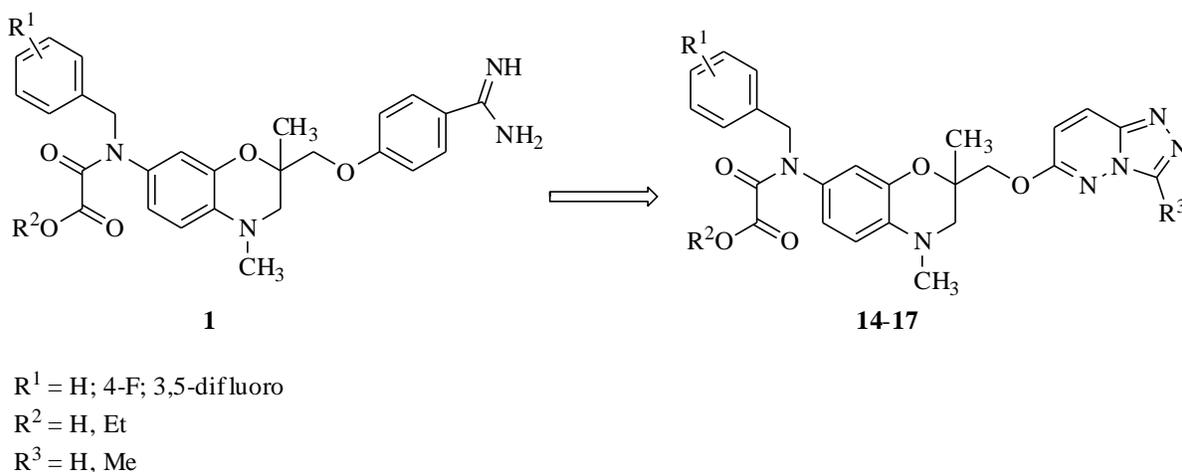
**Keywords:** [1,2,4]Triazolo[4,3-*b*]pyridazine, 2,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*][1,4]-oxazine, Mitsunobu reaction, benzamidine, thrombin inhibitor, cell proliferation

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## Introduction

Recently we have described a new series of potential antithrombotic compounds **1** based on a 2,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine scaffold possessing both thrombin inhibitory and fibrinogen receptor antagonistic activities.<sup>1</sup> In an attempt to study structure-activity relationships and improve both their thrombin inhibitory activity and fibrinogen receptor binding affinity, we systematically introduced fluorine into different positions of the P3 benzyl group and replaced the basic benzamidino group present in **1** with [1,2,4]triazolo[4,3-*b*]pyridazine-6-yl moiety that could mimic the benzamidino part of the molecule (Figure 1). It is well documented that aromatic carbon-bound fluorine can participate in hydrogen bonding and electrostatic interactions<sup>2</sup> thus contributing to the binding of drug molecules to their targets.<sup>3</sup> Since many

potent thrombin inhibitors possessing a highly basic benzamidine moiety lack oral bioavailability,<sup>4</sup> this functionality has frequently been replaced by less basic *N*-heterocycles such as imidazo[1,2-*a*]pyridine,<sup>5</sup> indole,<sup>6</sup> indazole,<sup>7</sup> and pyrrolo[3,2-*b*]pyridine<sup>8</sup> moieties. In our work, presented in this communication, introduction of fluorine into various positions of the aromatic ring of the P3 benzyl moiety improved the binding to both antithrombotic targets,<sup>9</sup> whereas replacement of the benzamidine functionality in **1** by [1,2,4]triazolo[4,3-*b*]pyridazine-6-yl group to obtain analogues **14-17**, had a detrimental effect on thrombin inhibition and platelet fibrinogen receptor binding. On the contrary, the antiproliferative activity of compounds **1** studied on two endothelial (HMEC-1, BAEC) and two cancer (HELA, MCF-7) cell lines in connection with their antiangiogenic potential related to their thrombin inhibitory and fibrinogen receptor antagonist activity, remained preserved in [1,2,4]triazolo[4,3-*b*]pyridazine compounds **14-17**.

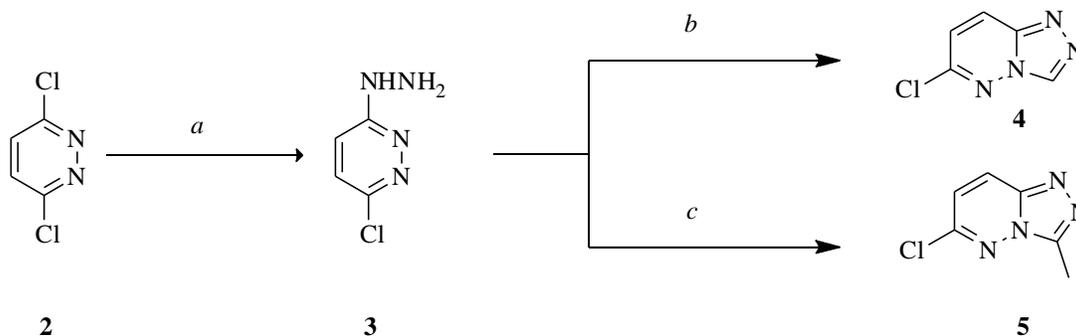


**Figure 1.** The general structure of compounds **14-17** derived from **1** by replacement of the benzamidine functionality with a [1,2,4]triazolo[4,3-*b*]pyridazine-6-yl moiety.

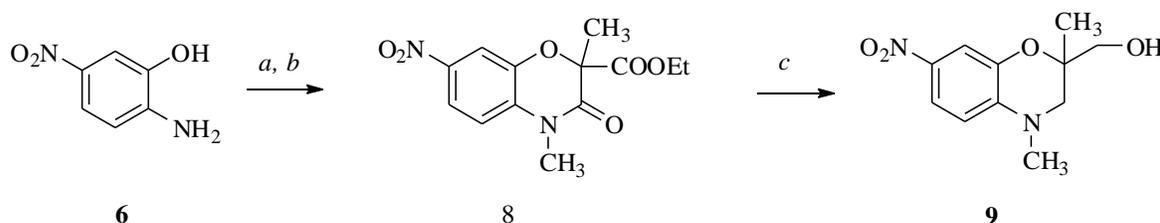
## Results and Discussion

The synthesis of target [1,2,4]triazolo[4,3-*b*]pyridazine compounds **14-17** is depicted in Scheme 3. The key intermediate 2-hydroxymethylbenzo[*b*][1,4]oxazine derivative **9** was prepared as described, from 2-amino-5-nitrophenol **6** in three steps via i) cyclization affording ethyl 2,4-dimethyl-7-nitro-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-2-carboxylate **7**, ii) its *N*-methylation to **8**<sup>10</sup> and iii) subsequent borane reduction<sup>1b</sup> of **8** (Scheme 2). 6-Chloro-[1,2,4]triazolo[4,3-*b*]pyridazine **4** was also obtained by a literature procedure, involving cyclization of 3-chloro-6-hydrazinylpyridazine **3** with diethyl ethoxymethylenemalonate,<sup>11</sup> and cyclization of **3** with triethyl orthoacetate afforded 6-chloro-3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazine **5**<sup>12</sup> (Scheme 1). As depicted in Scheme 3, substitution of chlorine in

[1,2,4]triazolo[4,3-*b*]pyridazines **4** and **5** with alcoholate of **9** obtained in situ with sodium hydride in *N,N*-dimethylformamide could be easily achieved to afford [1,2,4]triazolo[4,3-*b*]pyridazine-6-yl ethers **10a** and **10b**.



**Scheme 1.** Synthesis of compounds **4** and **5**. Reagents and conditions: *a*: hydrazine hydrate, 25% aq.  $\text{NH}_3$  (ref. 13); *b*:  $\text{EtOCH}=\text{C}(\text{COOEt})_2$ ,  $\text{CH}_3\text{CN}$ , reflux, 3 h; *c*:  $\text{CH}_3\text{C}(\text{OEt})_3$ , reflux, 12 h.



**Scheme 2.** Synthesis of **9**. Reagents and conditions: *a*:  $\text{Br}(\text{Me})\text{C}(\text{COOEt})_2$ ,  $\text{KF}$ ,  $\text{DMF}$ ,  $60^\circ\text{C}$ , 6 h; *b*:  $\text{MeI}$ , toluene, reflux, 2 h; *c*:  $\text{BH}_3\cdot\text{Me}_2\text{S}$ ,  $\text{THF}$ , reflux, 12 h.

After their reduction to amino derivatives **11a** and **11b**, *N*-benzylation at the aromatic amino group was carried out via imines formed with benzaldehyde, 4-fluorobenzaldehyde or 3,5-difluorobenzaldehyde, and their reduction using sodium borohydride to give compounds **12a-c** and **13a-c**. Acylation of amines **12** and **13** with ethyloxalyl chloride led to carboxamides **14a-c** and **15a-c**, respectively. Finally, hydrolysis of the ethyl esters with 1M lithium hydroxide gave the corresponding carboxylic acids **16a-c** and **17a-c**.

Compounds **14-17** were tested for thrombin inhibition according to a previously described protocol<sup>1b</sup> and found to be devoid of thrombin inhibitory activity ( $K_i > 300 \mu\text{M}$ ; results not shown) indicating that in this compound class substitution of the benzamidine with a [1,2,4]triazolo[4,3-*b*]pyridazin-6-yl moiety is detrimental for thrombin inhibition. Also the affinity for binding to platelet fibrinogen receptor tested according to a published procedure<sup>1b</sup> was lost ( $\text{IC}_{50} > 100 \mu\text{M}$ ; results not shown), indicating again that in this type of compound for preservation of fibrinogen receptor antagonistic activity the benzamidine moiety cannot be replaced by a [1,2,4]triazolo[4,3-*b*]pyridazine core.



7) were assessed. The results collected in Table 1 indicate that esters **14a-c** and **15a-c** inhibit the proliferation of both endothelial (HMEC and BAEC) cell lines and both carcinoma (HELA and MCF-7) cell lines, with IC<sub>50</sub> values ranging from 36.6 to 46.0 μM for HMEC-1, 33.5 to 38.0 μM for BAEC, 31.2 to 39.3 μM for HELA and 9.8 to 24.2 μM for MCF-7 cell lines, and were roughly 10-fold less potent than esters **1**.<sup>9</sup> A combination of ionic structures (amidinium ion) with lipophilic parts very often gives rise to inhibition of cell growth, which may account for the higher activity of the amidine derivatives in cell proliferation assays. The carboxylic acids **16a-c** and **17a-c** were devoid of anti-proliferative activity, suggesting their insufficient cellular uptake. Compounds **14a-c** and **15a-c** were weakly active in cell migration assay<sup>14</sup> but proved to be inactive in tube formation assay<sup>14</sup> and chorioallantoic membrane (CAM) assay<sup>15</sup> (data not shown), whereas carboxylic acids **16a-c** and **17a-c** did not show any activity in these three assays.

**Table 1.** Cytostatic activity of compounds **14** - **17** in endothelial cell lines (HMEC-1, BAEC) and cancer cell lines (HELA, MCF-7)

Compound	HMEC-1 (IC <sub>50</sub> , μM)	BAEC (IC <sub>50</sub> , μM)	HELA (IC <sub>50</sub> , μM)	MCF-7 (IC <sub>50</sub> , μM)
<b>14a</b>	46.0 ± 0.5	37.3 ± 2.5	36.9 ± 2.5	24.2 ± 3.5
<b>16a</b>	> 100	> 100	> 100	> 100
<b>14b</b>	45.3 ± 2.6	37.3 ± 3.0	37.0 ± 5.2	13.6 ± 1.9
<b>16b</b>	> 100	> 100	> 100	> 100
<b>14c</b>	39.1 ± 2.9	36.7 ± 3.6	34.2 ± 4.4	18.6 ± 1.3
<b>16c</b>	> 100	> 100	> 100	> 100
<b>15a</b>	44.7 ± 0.7	36.3 ± 4.8	39.3 ± 6.8	16.1 ± 0.7
<b>17a</b>	> 100	> 100	> 100	> 100
<b>15b</b>	38.4 ± 0.4	38.0 ± 2.3	31.2 ± 12.3	9.8 ± 1.8
<b>17b</b>	> 100	> 100	> 100	> 100
<b>15c</b>	36.6 ± 3.5	33.5 ± 6.9	33.7 ± 2.1	14.2 ± 3.0
<b>17c</b>	> 100	> 100	>100	> 100

## Conclusions

Compounds **14** - **17** were synthesized as analogues of potential dual antithrombotic compounds **1** possessing thrombin inhibitory and fibrinogen receptor antagonistic activity. The replacement of the benzamidine functionality of compounds **1** with a [1,2,4]triazolo[4,3-*b*]pyridazine moiety resulted in loss of thrombin inhibitory and fibrinogen receptor antagonistic activity. However, anti-proliferative activity against HMEC-1, BAEC, HELA and MCF-7 cell lines inherent to

benzamidines **1** was retained in esters **14a-c** and **15a-c**, indicating that this activity may not be attributed to the presence of a basic benzamidino group.

## Experimental Section

**General.** Chemicals were obtained from Acros, Sigma-Aldrich, Fluka and Alfa Aesar and used without further purification. Hydrogenation reactions were carried out using a Parr 4842 hydrogenation apparatus. Analytical TLC was performed on silica gel Merck 60 F<sub>254</sub> plates (0.25 mm), using visualization with ultraviolet light. Column chromatography was carried out on silica gel 60 (particle size 240-400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra were recorded on a Bruker AVANCE III spectrometer (400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C nuclei) in DMSO-*d*<sub>6</sub> solution with TMS as the internal standard. The coupling constants (*J*) are given in Hz, and the splitting patterns are appointed as: s (singlet), d (doublet), dd (double doublet), t (triplet) and m (multiplet). IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Microanalyses were performed at University of Ljubljana, Faculty of Chemistry and Chemical Technology on a Perkin-Elmer C, H, N analyzer 240 C. Mass spectra were obtained at Jožef Stefan Institute, Ljubljana, using a VG-Analytical Autospec Q mass spectrometer. HPLC analyses were performed on an Agilent Technologies HP 1100 instrument with G1365B UV-VIS detector (254 nm), using an Eclips Plus C18 column (4.6 × 150 mm) at flow rate 1 mL/min. The eluant was a mixture of 0.1 % TFA in water (A) and methanol (B). Gradient was 40% B to 80% B in 15 minutes. Purification of final compounds, if necessary, was performed by reverse phase column chromatography using a Flash Purification System ISOLERA<sup>TM</sup> with 0.1 % TFA in water and methanol as an eluent. Synthesis of compounds **7**, **8** and **9<sup>1b</sup>** and [1,2,4]triazolo[4,3-*b*]pyridazine building blocks **4-6**<sup>11-13</sup> were performed according to published synthetic procedures.

### General procedure for the synthesis of compounds (10a) and (10b)

Compound **9** (7.70 g, 32.3 mmol) was dissolved in 100 ml absolute DMF and 60% NaH (1.4 g, 58.1 mmol) was added. The mixture was stirred for 1 h at room temperature under argon atmosphere, whereupon 6-chloro-[1,2,4]triazolo[4,3-*b*]pyridazine **4** (5.0 g, 32.3 mmol) was added. The mixture was stirred for 2 h at room temperature, then at 110 °C overnight. Product **10a** was purified by column chromatography using DCM/MeOH 20:1 as eluent. to afford 10.1 g (88% yield) of **10a** of yellow powder.

**2-((1,2,4)Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-7-nitro-3,4-dihydro-2H-1,4-benzoxazine (10a).** Yellow powder, yield: 88%; mp 166-168 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.43 (s, 3H, 2-CH<sub>3</sub>), 3.08 (s, 3H, *N*-CH<sub>3</sub>), 3.37-3.41 (d, overlapped with water, 1H, 3-H), 3.61 (d, *J* 12.6 Hz, 1H, 3-H), 4.37 (d, *J* 11.3 Hz, 1H, CH<sub>2</sub>O), 4.40 (d, *J* 11.2 Hz, 1H, CH<sub>2</sub>O), 6.85 (d, *J* 9.2 Hz, 1H, Ar-H<sup>5</sup>), 7.13 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.48 (d, *J* 2.6 Hz, 1H, Ar-

H<sup>8</sup>), 7.80 (dd, *J* 9.1, 2.6 Hz, 1H, Ar-H<sup>6</sup>), 8.30 (dd, *J* 9.8, 0.8 Hz, 1H, Ar-H<sup>8</sup>), 9.39 (d, *J* 0.7 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 143.0, 141.7, 140.7, 139.4, 137.1, 127.1, 119.5, 117.1, 111.3, 110.6, 73.9, 69.9, 53.3, 38.6, 31.2, 20.8; HRMS (ESI) *m/z* calcd for C<sub>16</sub>H<sub>17</sub>N<sub>6</sub>O<sub>4</sub> [M + H]<sup>+</sup> 357.1311, found 357.1312; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3447, 3129, 1601, 1497, 1302, 1018, 830; HPLC: 98.4 %, *t*<sub>r</sub> 11.6 min; Anal. Calcd. for C<sub>16</sub>H<sub>16</sub>N<sub>6</sub>O<sub>4</sub> × 1/6 H<sub>2</sub>O: C, 53.45; H, 4.50; N, 23.58. Found: C, 53.48; H, 4.58; N, 23.39%.

**2,4-Dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-7-nitro-3,4-dihydro-2*H*-1,4-benzoxazine (10b).** Yellow powder, yield: 84%; mp 121-123 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.42 (s, 3H, 2-CH<sub>3</sub>), 2.52 (s, 3H, 3'-CH<sub>3</sub>), 3.07 (s, 3H, *N*-CH<sub>3</sub>), 3.36-3.40 (d, overlapped with water, 1H, 3-H), 3.60 (d, *J* 12.6 Hz, 1H, 3-H), 4.40 (d, *J* 11.3 Hz, 1H, CH<sub>2</sub>O), 4.47 (d, *J* 11.3 Hz, 1H, CH<sub>2</sub>O), 6.83 (d, *J* 9.2 Hz, 1H, Ar-H<sup>5</sup>), 7.05 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.42 (d, *J* 2.6 Hz, 1H, Ar-H<sup>8</sup>), 7.78 (dd, *J* 9.1, 2.6 Hz, 1H, Ar-H<sup>6</sup>), 8.23 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 160.4, 146.4, 143.2, 141.8, 140.8, 137.0, 127.2, 119.4, 116.0, 111.3, 110.6, 74.1, 69.6, 43.3, 38.6, 20.9, 9.6; HRMS (ESI) *m/z* calcd for C<sub>17</sub>H<sub>19</sub>N<sub>6</sub>O<sub>4</sub> [M + H]<sup>+</sup> 371.1468, found 371.1462; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3606, 3340, 1599, 1502, 1291, 1073, 879; HPLC: 100 %, *t*<sub>r</sub> 12.3 min; Anal. Calcd. for C<sub>17</sub>H<sub>18</sub>N<sub>6</sub>O<sub>4</sub> × 1/2 H<sub>2</sub>O: C, 53.53; H, 5.21; N, 22.23. Found: C, 53.82; H, 5.05; N, 22.15%.

#### General procedure for reduction of compounds (10a) and (10b) by catalytic hydrogenation

The hydrogenation reaction was performed in a hydrogenator (25 bar, 1 h, 10% Pd/C). A mixture of compound **10a** (9.0 g, 24.3 mmol) dissolved in 100 ml MeOH and 10% Pd/C (0.9 g) was stirred at room temperature till the reaction was completed (1 h). The catalyst was filtered off and the solvent was evaporated in vacuo to yield 8.3 g (100%) of **11a**.

**2-(((1,2,4]Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (11a)** and **2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (11b)** were used in the next step without further purification.

#### General procedure for reductive amination (aldimine formation/reduction) of compounds (11a) and (11b)

Benzaldehyde (2.4 g, 22.3 mmol), amine **11a** (8 g, 24.6 mmol) and molecular sieves (0.3 nm) were mixed in 100 ml absolute methanol at room temperature under argon atmosphere. The mixture was stirred overnight until the aldimine formation was complete. The aldimine in methanol was carefully treated with solid NaBH<sub>4</sub> (1.6 eq, 35.7 mmol, 1.3 g) and the reaction mixture was stirred for additional 2 h. The solvent was removed in vacuo and the crude product isolated by extraction with dichloromethane (DCM). The product was purified by column chromatography using DCM/MeOH 20:1 as eluent to afford **12a** as yellow powder (5.2 g, yield 51%).

**2-(((1,2,4]Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-*N*-benzyl-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (12a).** Yellow powder, yield 5.2 g (51%); mp 69-71 °C; <sup>1</sup>H NMR (400

MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.39 (s, 3H, 2-CH<sub>3</sub>), 2.70 (s, 3H, *N*-CH<sub>3</sub>), 2.84 (d, *J* 9.8 Hz, 1H, 3-H), 3.15 (d, *J* 9.9 Hz, 1H, 3-H), 4.15 (s, 2H, Ph-CH<sub>2</sub>), 4.32 (s, 2H, CH<sub>2</sub>O), 5.70 (s, 1H, NH), 5.96-6.23 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.55 (s, 1H, Ar-H<sup>8</sup>), 7.13 (d, *J* 9.9 Hz, 1H, Ar-H<sup>7</sup>), 7.16-7.38 (m, 5H, Ph), 8.28 (dd, *J* 9.8, 0.8 Hz, 1H, Ar-H<sup>8</sup>), 9.39 (d, *J* 0.8 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  160.9, 143.0, 142.1, 139.4, 138.1, 137.7, 132.8, 128.7, 127.6, 127.0, 126.9, 126.7, 125.4, 122.4, 117.2, 115.7, 110.2, 76.4, 62.6, 53.8, 38.0, 20.7, 13.3; HRMS (ESI) *m/z* calcd for C<sub>23</sub>H<sub>24</sub>N<sub>6</sub>O<sub>2</sub> [M]<sup>+</sup> 416.1961, found 416.1960; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3392, 2808, 1627, 1518, 1297, 1019, 819; HPLC: 97.9%, *t*<sub>r</sub> 8.4 min.

**2-(((1,2,4)Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-*N*-(4-fluorobenzyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (12b).** Yellow powder, yield 5.8 g (54%); mp 66-68 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.39 (s, 3H, 2-CH<sub>3</sub>), 2.70 (s, 3H, *N*-CH<sub>3</sub>), 2.84 (d, *J* 9.9 Hz, 1H, 3-H), 3.16 (d, *J* 9.8 Hz, 1H, 3-H), 4.12 (s, 2H, Ph-CH<sub>2</sub>), 4.32 (d, *J* 11.0 Hz, 1H, CH<sub>2</sub>O), 4.44 (d, *J* 11.0 Hz, 1H, CH<sub>2</sub>O), 5.72 (s, 1H, NH), 5.89-6.20 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.55 (s, 1H, Ar-H<sup>8</sup>), 7.05-7.17 (m, 3H, Ph, Ar-H<sup>7</sup>), 7.28-7.40 (m, 2H, Ph), 8.28 (dd, *J* 9.8, 0.8 Hz, 1H, Ar-H<sup>8</sup>), 9.39 (d, *J* 0.8 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  162.6, 160.9, 160.2, 146.2, 143.0, 139.4, 129.4, 129.4, 127.0, 117.2, 115.5, 115.3, 111.2, 111.0, 103.4, 103.1, 102.8, 74.1, 61.1, 53.1, 50.2, 38.0, 20.7; HRMS (ESI) *m/z* calcd for C<sub>23</sub>H<sub>23</sub>FN<sub>6</sub>O<sub>2</sub> [M]<sup>+</sup> 434.1867, found 434.1868; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3344, 3064, 2810, 1627, 1508, 1333, 1217, 1016, 819; HPLC: 96.2%, *t*<sub>r</sub> 8.8 min.

**2-(((1,2,4)Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-*N*-(3,5-difluorobenzyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (12c).** Yellow powder, yield 5.4 g (49%); mp 71-73 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.39 (s, 3H, 2-CH<sub>3</sub>), 2.71 (s, 3H, *N*-CH<sub>3</sub>), 2.86 (d, *J* 9.8 Hz, 1H, 3-H), 3.16 (d, *J* 9.8 Hz, 1H, 3-H), 4.19 (s, 2H, Ph-CH<sub>2</sub>), 4.32 (s, 2H, CH<sub>2</sub>O), 5.86 (s, 1H, NH), 5.92-6.19 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.56 (d, *J* 6.3 Hz, 1H, m, 2H, Ar-H<sup>8</sup>), 7.00-7.08 (m, 3H, Ph), 7.13 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 8.28 (dd, *J* 9.8, 0.8 Hz, 1H, Ar-H<sup>8</sup>), 9.39 (d, *J* 0.8 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  162.3, 160.9, 146.3, 142.9, 139.4, 129.3, 129.1, 127.0, 117.2, 115.1, 115.0, 111.9, 111.1, 104.1, 103.9, 102.8, 74.0, 62.5, 60.1, 53.8, 38.2, 20.7, 13.3; HRMS (ESI) *m/z* calcd for C<sub>23</sub>H<sub>23</sub>F<sub>2</sub>N<sub>6</sub>O<sub>2</sub> [M + H]<sup>+</sup> 453.1851, found 453.1840; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3380, 2810, 1630, 1515, 1333, 1302, 1179, 1020, 818; HPLC: 96.8%, *t*<sub>r</sub> 9.0 min.

***N*-Benzyl-2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (13a):** Yellow powder, yield 5.2 g (51%); mp 81-83 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.39 (s, 3H, 2-CH<sub>3</sub>), 2.52 (s, 3H, 3'-CH<sub>3</sub>), 2.85 (d, *J* 11.0 Hz, 1H, 3-H), 3.16 (d, *J* 11.1 Hz, 1H, 3-H), 3.38 (s, 3H, *N*-CH<sub>3</sub>); 4.11 (s, 2H, Ph-CH<sub>2</sub>), 4.31 (d, *J* 10.4 Hz, 1H, CH<sub>2</sub>O), 4.43 (d, *J* 10.3 Hz, 1H, CH<sub>2</sub>O), 5.69 (s, 1H, NH), 5.84-6.21 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.55 (s, 1H, Ar-H<sup>8</sup>), 7.06 (d, *J* 9.8 Hz, 1H, , Ar-H<sup>7</sup>), 7.16-7.36 (m, 5H, Ph), 8.21 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>):  $\delta$  160.5, 160.2, 146.6, 143.7, 143.2, 141.9, 137.0, 128.7, 127.6, 127.0, 126.9, 116.1, 116.0, 115.5, 114.9, 114.0, 106.1, 102.8, 74.8, 69.2, 55.9, 47.0, 21.7, 9.7; HRMS (ESI) *m/z* calcd for C<sub>24</sub>H<sub>27</sub>N<sub>6</sub>O<sub>2</sub> [M + H]<sup>+</sup> 431.2198, found 431.2178; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3385, 3057, 2808, 1627, 1519, 1299, 1180, 1017, 818; HPLC: 96.4%, *t*<sub>r</sub> 9.3 min.

***N*-(4-Fluorobenzyl)-2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)-methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (13b).** Yellow powder, yield 5.1 g (48%);

mp 80-82 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.39 (s, 3H, 2-CH<sub>3</sub>), 2.70 (s, 3H, 3'-CH<sub>3</sub>), 2.84 (d, *J* 11.5 Hz, 1H, 3-H), 3.16 (d, *J* 11.4 Hz, 1H, 3-H), 3.38 (s, 3H, *N*-CH<sub>3</sub>), 4.09 (d, *J* 5.3 Hz, 2H, Ph-CH<sub>2</sub>), 4.31 (d, *J* 10.9 Hz, 1H, CH<sub>2</sub>O), 4.43 (d, *J* 10.9 Hz, 1H, CH<sub>2</sub>O), 5.69 (t, *J* 5.7 Hz, 1H, NH), 5.94 (d, *J* 2.4 Hz, 1H, Ar-H<sup>5</sup>), 6.12 (dd, *J* 8.6, 2.5 Hz, 1H, Ar-H<sup>6</sup>), 6.56 (d, *J* 8.6 Hz, 1H, Ar-H<sup>8</sup>), 7.06 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.09-7.16 (m, 2H, Ph), 7.27-7.38 (m, 2H, Ph), 8.21 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 162.6, 160.5, 160.2, 146.6, 144.1, 143.2, 142.7, 137.3, 129.4, 129.3, 127.1, 126.7, 116.1, 115.5, 115.3, 114.4, 106.2, 101.3, 75.0, 70.2, 55.5, 46.8, 21.7, 9.7; HRMS (ESI) *m/z* calcd for C<sub>24</sub>H<sub>26</sub>FN<sub>6</sub>O<sub>2</sub> [M + H]<sup>+</sup> 449.2101, found 449.2110; IR (KBr) ν<sub>max</sub>/cm<sup>-1</sup>: 3368, 3064, 2810, 1625, 1517, 1300, 1094, 1014, 820; HPLC: 95.7%, t<sub>r</sub> 9.7 min.

***N*-(3,5-Difluorobenzyl)-2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)-methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-amine (13c)**. Yellow powder, yield 6.0 g (51%); mp 80-82 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.39 (s, 3H, 2-CH<sub>3</sub>), 2.71 (s, 3H, 3'-CH<sub>3</sub>), 2.86 (d, *J* 11.5 Hz, 1H, 3-H), 3.17 (d, *J* 11.5 Hz, 1H, 3-H), 3.38 (s, 3H, *N*-CH<sub>3</sub>), 4.16 (d, *J* 5.5 Hz, 2H, Ph-CH<sub>2</sub>), 4.32 (d, *J* 10.9 Hz, 1H, CH<sub>2</sub>O), 4.42 (d, *J* 10.9 Hz, 1H, CH<sub>2</sub>O), 5.83 (t, *J* 6.0 Hz, 1H, NH), 5.96 (d, *J* 2.4 Hz, 1H, Ar-H<sup>5</sup>), 6.10 (dd, *J* 8.6, 2.4 Hz, 1H, Ar-H<sup>6</sup>), 6.56 (d, *J* 8.7 Hz, 1H, Ar-H<sup>8</sup>), 6.96-7.11 (m, 4H, Ph, Ar-H<sup>7</sup>), 8.21 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 160.5, 146.6, 144.1, 143.2, 142.2, 127.1, 126.9, 116.1, 114.5, 110.4, 110.4, 110.3, 110.2, 106.2, 102.6, 102.3, 102.1, 101.4, 75.0, 70.3, 55.4, 46.7, 21.7, 9.7; HRMS (ESI) *m/z* calcd for C<sub>24</sub>H<sub>25</sub>F<sub>2</sub>N<sub>6</sub>O<sub>2</sub> [M + H]<sup>+</sup> 467.2007, found 467.2004; IR (KBr) ν<sub>max</sub>/cm<sup>-1</sup>: 3385, 3056, 2812, 1624, 1518, 1302, 1188, 1016, 819; HPLC: 98.0%, t<sub>r</sub> 10.8 min.

### General procedure for preparation of *N*-acylated compounds (14a-c) and (15a-c)

Ethyl oxalyl chloride (1.6 g, 11.5 mmol) was added to a solution of compound **12a** (4.0 g, 9.6 mmol) and triethylamine (1.2 g, 11.5 mmol) in dichloromethane (70 ml) and the mixture stirred for 2 h. The solvent was removed under reduced pressure, the residue dissolved in ethyl acetate (50 ml) and washed successively with a 10% citric acid solution (3 x 50 ml), saturated NaHCO<sub>3</sub> solution (3 x 50 ml) and brine (1 x 50 ml). The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub> and the solvent evaporated under reduced pressure. The oily product was purified by column chromatography using petroleum ether/ethyl acetate (1.5:1) as eluent to afford **14a** as a white powder (2.9 g, yield 58%).

**Ethyl 2-((2-(((1,2,4]triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(benzyl)amino)-2-oxoacetate (14a)**. White powder, yield 2.9 g (58%); mp 70-72 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 0.80 (t, *J* 7.1 Hz, 3H, CH<sub>2</sub>CH<sub>3</sub>), 1.39 (s, 3H, 2-CH<sub>3</sub>), 2.84 (s, 3H, *N*-CH<sub>3</sub>), 3.08 (d, *J* 11.9 Hz, 1H, 3-H), 3.34 (d, *J* 11.9 Hz, 1H, 3-H), 3.92 (q, *J* 7.1 Hz, 2H, CH<sub>2</sub>CH<sub>3</sub>), 4.32 (d, *J* 11.1 Hz, 1H, CH<sub>2</sub>O), 4.28 (d, *J* 11.1 Hz, 1H, CH<sub>2</sub>O), 4.84 (s, 2H, Ph-CH<sub>2</sub>), 6.52-6.59 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.67 (d, *J* 8.4 Hz, 1H, Ar-H<sup>8</sup>), 7.11 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.14-7.34 (m, 5H, Ph), 8.29 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>), 9.40 (s, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 162.6, 161.7, 160.4, 142.5, 141.9, 138.9, 136.3, 135.2, 129.0, 128.5, 127.8, 127.4, 126.5, 120.5, 116.6, 115.4, 115.2, 114.8, 111.8, 74.0, 69.8, 61.0, 53.3, 51.0, 38.0, 20.5, 13.3;

HRMS (ESI)  $m/z$  calcd for  $C_{27}H_{29}N_6O_5$   $[M + H]^+$  517.2199, found 517.2207; IR (KBr)  $\nu_{max}/cm^{-1}$ : 3447, 2980, 1740, 1663, 1518, 1298, 1193, 1021, 818; HPLC: 100 %,  $t_r$  15.3 min; Anal. Calcd. for  $C_{27}H_{28}N_6O_5 \times \frac{1}{2} H_2O$ : C, 61.91; H, 5.48; N, 15.99. Found: C, 61.70; H, 5.56; N, 15.99%.

**Ethyl 2-((2-((1,2,4)triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(4-fluorobenzyl)amino)-2-oxoacetate (14b).** White powder, yield 2.6 g (52%); mp 73-75 °C;  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.72 (t,  $J$  7.1 Hz, 3H,  $CH_2CH_3$ ), 1.32 (s, 3H, 2- $CH_3$ ), 2.78 (s, 3H, *N*- $CH_3$ ), 3.01 (d,  $J$  11.9 Hz, 1H, 3-H), 3.27 (d, overlapped with water, 1H, 3-H), 3.84 (q,  $J$  7.1 Hz, 2H,  $CH_2CH_3$ ), 4.22 (d,  $J$  11.1 Hz, 1H,  $CH_2O$ ), 4.26 (d,  $h$  11.1 Hz, 1H,  $CH_2O$ ), 4.76 (s, 2H, Ph- $CH_2$ ), 6.44-6.51 (m, 2H, Ar- $H^5$ , Ar- $H^6$ ), 6.60 (d,  $J$  8.3 Hz, 1H, Ar- $H^8$ ), 7.02-7.11 (m, 3H, Ph- $H^3$ ,  $H^5$ , Ar- $H^7$ ), 7.11-7.17 (m, 2H, Ph- $H^2$ ,  $H^6$ ), 8.22 (d,  $J$  9.8 Hz, 1H, Ar- $H^8$ ), 9.34 (s, 1H, Ar- $H^3$ );  $^{13}C$  NMR (DMSO- $d_6$ ):  $\delta$  162.5, 161.7, 160.4, 142.5, 141.9, 138.9, 135.2, 132.6, 132.5, 130.0, 129.9, 128.7, 126.6, 120.6, 116.6, 115.4, 115.2, 114.8, 111.8, 73.9, 69.8, 61.0, 53.3, 50.1, 38.0, 20.5, 13.3; HRMS (ESI)  $m/z$  calcd for  $C_{27}H_{28}FN_6O_5$   $[M + H]^+$  535.2105, found 535.2118; IR (KBr)  $\nu_{max}/cm^{-1}$ : 3447, 2981, 1741, 1664, 1509, 1219, 1022, 820; HPLC: 100 %,  $t_r$  15.6 min; Anal. Calcd. for  $C_{27}H_{27}FN_6O_5 \times \frac{1}{2} H_2O$ : C, 59.87; H, 5.14; N, 15.21. Found: C, 59.66; H, 5.19; N, 15.46%.

**Ethyl 2-((2-((1,2,4)triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(3,5-difluorobenzyl)amino)-2-oxoacetate (14c).** White powder, yield 2.7 g (56%); mp 71-73 °C;  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.81 (t,  $J$  7.1 Hz, 3H,  $CH_2CH_3$ ), 1.40 (s, 3H, 2- $CH_3$ ), 2.86 (s, 3H, *N*- $CH_3$ ), 3.10 (d,  $J$  11.9 Hz, 1H, 3-H), 3.35 (d, overlapped with water, 1H, 3-H), 3.94 (q,  $J$  7.1 Hz, 2H,  $CH_2CH_3$ ), 4.29 (d,  $J$  11.1 Hz, 1H,  $CH_2O$ ), 4.34 (d,  $J$  11.1 Hz, 1H,  $CH_2O$ ), 4.88 (s, 2H, Ph- $CH_2$ ), 6.58-6.64 (m, 2H, Ar- $H^5$ , Ar- $H^6$ ), 6.70 (d,  $J$  8.4 Hz, 1H, Ar- $H^8$ ), 6.86-6.92 (m, 2H, Ph), 7.13 (d,  $J$  9.9 Hz, 1H, Ar- $H^7$ ), 7.12-7.19 (m, 1H, Ph), 8.30 (dd,  $J$  9.9, 0.8 Hz, 1H, Ar- $H^8$ ), 9.43 (d,  $J$  0.7 Hz, 1H, Ar- $H^3$ );  $^{13}C$  NMR (DMSO- $d_6$ ):  $\delta$  162.4, 161.8, 160.4, 142.5, 142.0, 139.0, 135.3, 128.7, 126.6, 120.4, 116.6, 114.5, 111.9, 111.0, 110.9, 110.9, 110.8, 74.0, 69.8, 64.9, 61.2, 53.3, 50.1, 38.0, 20.5, 15.2, 13.3; HRMS (ESI)  $m/z$  calcd for  $C_{27}H_{27}F_2N_6O_5$   $[M + H]^+$  553.2011, found 553.2036; IR (KBr)  $\nu_{max}/cm^{-1}$ : 3445, 2980, 1733, 1653, 1510, 1300, 1189, 1025, 818; HPLC: 97.1 %,  $t_r$  16.5 min. Anal. Calcd. for  $C_{27}H_{26}F_2N_6O_5 \times \frac{1}{3} H_2O$ : C, 58.09; H, 4.75; N, 14.90. Found: C, 58.06; H, 4.81; N, 15.05%.

**Ethyl 2-(benzyl(2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetate (15a).** White powder, yield 2.9 g (60%); mp 75-77 °C;  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.84 (t,  $J$  7.1 Hz, 3H,  $CH_2CH_3$ ), 1.38 (s, 3H, 2- $CH_3$ ), 2.57 (s, 3H, 3'- $CH_3$ ), 2.84 (s, 3H, *N*- $CH_3$ ), 3.09 (d,  $J$  11.9 Hz, 1H, 3-H), 3.33 (d, overlapped with water, 1H, 3-H), 3.95 (q,  $J$  7.1 Hz, 2H,  $CH_2CH_3$ ), 4.36 (d,  $J$  11.1 Hz, 1H,  $CH_2O$ ), 4.33 (d,  $J$  11.0 Hz, 1H,  $CH_2O$ ), 4.84 (s, 2H, Ph- $CH_2$ ), 6.52-6.60 (m, 2H, Ar- $H^5$ , Ar- $H^6$ ), 6.68 (d,  $J$  8.5 Hz, 1H, Ar- $H^8$ ), 7.05 (d,  $J$  9.8 Hz, 1H, Ar- $H^7$ ), 7.12-7.37 (m, 5H, Ph), 8.22 (d,  $J$  9.8 Hz, 1H, Ar- $H^8$ );  $^{13}C$  NMR (DMSO- $d_6$ ):  $\delta$  162.6, 161.7, 159.9, 146.0, 142.7, 141.9, 136.3, 135.2, 129.0, 128.5, 127.8, 127.4, 126.7, 120.5, 115.5, 114.7, 111.9, 74.1, 69.5, 61.0, 53.2, 50.8, 38.1, 20.7, 13.3, 9.2 (two more peaks overlapped with DMSO); HRMS (ESI)  $m/z$  calcd for  $C_{28}H_{31}N_6O_5$   $[M + H]^+$  531.2356, found 531.2345; IR (KBr)  $\nu_{max}/cm^{-1}$ : 3435, 2980, 1741, 1664,

1518, 1334, 1193, 1021, 816; HPLC: 100%,  $t_r$  16.0 min; Anal. Calcd. for  $C_{28}H_{30}N_6O_5$ : C, 63.06; H, 5.70; N, 15.60. Found: C, 63.38; H, 5.70; N, 15.84%.

**Ethyl 2-((2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(4-fluorobenzyl)amino)-2-oxoacetate (15b).** White powder, yield 2.5 g (51%); mp 74-76 °C;  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.83 (t,  $J$  7.1 Hz, 3H,  $CH_2CH_3$ ), 1.39 (s, 3H, 2- $CH_3$ ), 2.57 (s, 3H, 3'- $CH_3$ ), 2.85 (s, 3H, *N*- $CH_3$ ), 3.10 (d,  $J$  11.9 Hz, 1H, 3-H), 3.33 (d, overlapped with water, 1H, 3-H) 3.94 (q,  $J$  7.1 Hz, 2H,  $CH_2CH_3$ ), 4.37 (d,  $J$  11.0 Hz, 1H,  $CH_2O$ ), 4.33 (d,  $J$  11.0 Hz, 1H,  $CH_2O$ ), 4.83 (s, 2H, Ph- $CH_2$ ), 6.52-6.57 (m, 2H, Ar- $H^5$ , Ar- $H^6$ ), 6.68 (d,  $J$  9.2 Hz, 1H, Ar- $H^8$ ), 7.05 (d,  $J$  9.8 Hz, 1H, Ar- $H^7$ ), 7.11-7.24 (m, 4H, Ph), 8.23 (d,  $J$  9.8 Hz, 1H, Ar- $H^8$ );  $^{13}C$  NMR (DMSO- $d_6$ ):  $\delta$  162.6, 161.7, 160.0, 146.0, 142.7, 141.9, 135.3, 132.6, 132.5, 130.0, 129.9, 128.8, 126.7, 120.6, 115.5, 115.4, 115.2, 114.8, 111.9, 74.1, 69.5, 61.0, 53.2, 50.0, 38.0, 20.7, 13.3, 9.2; HRMS (ESI)  $m/z$  calcd for  $C_{28}H_{30}FN_6O_5$  [ $M + H$ ] $^+$  549.2262, found 549.2270; IR (KBr)  $\nu_{max}/cm^{-1}$ : 3422, 3071, 2981, 1741, 1664, 1511, 1194, 1021, 816; HPLC: 100%,  $t_r$  16.0 min Anal. Calcd. for  $C_{28}H_{29}FN_6O_5 \times \frac{1}{2} H_2O$ : C, 60.48; H, 5.35; N, 15.01. Found: C, 60.31; H, 5.42; N, 15.07%.

**Ethyl 2-((3,5-difluorobenzyl)(2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetate (15c).** White powder, yield 2.9 g (59%); mp 77-79 °C;  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  0.85 (t,  $J$  7.1 Hz, 3H,  $CH_2CH_3$ ), 1.40 (s, 3H, 2- $CH_3$ ), 2.57 (s, 3H, 3'- $CH_3$ ), 2.86 (s, 3H, *N*- $CH_3$ ), 3.10 (d,  $J$  11.9 Hz, 1H, 3-H), 3.32-3.35 (d, overlapped with water, 1H, 3-H), 3.97 (q,  $J$  7.1 Hz, 2H,  $CH_2CH_3$ ), 4.34 (d,  $J$  11.0 Hz, 1H,  $CH_2O$ ), 4.38 (d,  $J$  11.1 Hz, 1H,  $CH_2O$ ), 4.87 (s, 2H, Ph- $CH_2$ ), 6.59-6.64 (m, 2H, Ar- $H^5$ , Ar- $H^6$ ), 6.66-6.73 (m, 1H, Ar- $H^8$ ), 6.90 (d,  $J$  6.2 Hz, 2H, Ph), 7.05 (d,  $J$  9.8 Hz, 1H, Ar- $H^7$ ), 7.11-7.18 (m, 1H, Ph), 8.22 (d,  $J$  9.8 Hz, 1H, Ar- $H^8$ );  $^{13}C$  NMR (DMSO- $d_6$ ):  $\delta$  162.4, 161.8, 160.0, 146.0, 142.1, 135.4, 128.7, 126.7, 120.4, 115.4, 114.5, 111.9, 111.0, 110.9, 110.8, 110.7, 103.3, 103.0, 102.8, 74.1, 69.5, 61.1, 53.2, 50.2, 38.1, 20.7, 13.3, 9.2; HRMS (ESI)  $m/z$  calcd for  $C_{28}H_{29}F_2N_6O_5$  [ $M + H$ ] $^+$  567.2167, found 567.2173; IR (KBr)  $\nu_{max}/cm^{-1}$ : 3422, 3078, 2981, 1743, 1667, 1518, 1301, 1200, 1029, 815; HPLC: 98.5%,  $t_r$  16.9 min; Anal. Calcd. for  $C_{28}H_{28}F_2N_6O_5$ : C, 59.23; H, 5.06; N, 14.61. Found: C, 59.36; H, 4.98; N, 14.83%.

### General procedure for alkaline hydrolysis of ethyl esters (14a-c) and (15a-c)

To the solution of ester **14a** (1 g, 1.94 mmol) in tetrahydrofuran (6 mL) and methanol (2 ml), 1M LiOH (11.6 ml, 11.6 mmol) was added and the reaction mixture stirred at room temperature for 2 hours. Solvent was evaporated under vacuum and the resulting aqueous solution neutralized with 1M hydrochloric acid until the product started to precipitate. The product was filtered to obtain **16a** as a pale purple powder (521 mg, yield 55%).

**2-(((1,2,4]Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(benzyl)amino)-2-oxoacetic acid (16a).** Pale purple powder, yield 521 mg (55%); mp 130-132 °C;  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  1.39 (s, 3H, 2- $CH_3$ ), 2.84 (s, 3H, *N*- $CH_3$ ), 3.08 (d,  $J$  11.8 Hz, 1H, 3-H), 3.29-3.35 (d, overlapped with water, 1H, 3-H), 4.30 (d,  $J$  11.0 Hz, 1H,  $CH_2O$ ), 4.35 (d,  $J$  11.0 Hz, 1H,  $CH_2O$ ), 4.82 (d,  $J$  2.9 Hz, 2H, Ph- $CH_2$ ), 6.57-6.63

(m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.64-6.69 (m, 1H, Ar-H<sup>8</sup>), 7.12 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.15-7.35 (m, 5H, Ph), 8.30 (dd, *J* 9.8, 0.8 Hz, 1H, Ar-H<sup>8</sup>), 9.41 (d, *J* 0.8 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 164.3, 163.2, 160.4, 142.5, 141.9, 138.9, 136.6, 135.0, 129.6, 128.4, 127.7, 127.2, 126.5, 120.5, 116.6, 114.6, 111.8, 74.1, 69.9, 61.1, 53.3, 50.7, 38.0, 20.7, 13.3; HRMS (ESI) *m/z* calcd for C<sub>25</sub>H<sub>25</sub>N<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup> 489.1886, found 489.1876; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3422, 3065, 2937, 1734, 1654, 1518, 1300, 1218, 1021, 821; HPLC: 92.0%, *t*<sub>r</sub> 12.6 min.

**2-((2-((1,2,4)Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(4-fluorobenzyl)amino)-2-oxoacetic acid (16b).** Pale purple powder, yield 483 mg (51%); mp 125-127 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.39 (s, 3H, 2-CH<sub>3</sub>), 2.85 (s, 3H, *N*-CH<sub>3</sub>), 3.08 (d, *J* 11.9 Hz, 1H, 3-H), 3.31-3.36 (d, overlaped with water, 1H, 3-H), 4.30 (d, *J* 11.0 Hz, CH<sub>2</sub>O), 4.35 (d, *J* 11.0 Hz, 1H, CH<sub>2</sub>O), 4.81 (s, 2H, Ph-CH<sub>2</sub>), 6.55-6.60 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.67 (dd, *J* 7.7, 1.3 Hz, 1H, Ar-H<sup>8</sup>), 7.08-7.16 (m, 3H, Ph, Ar-H<sup>7</sup>), 7.17-7.23 (m, 2H, Ph), 8.30 (dd, *J* 9.8, 0.8 Hz, 1H, Ar-H<sup>8</sup>), 9.41 (d, *J* 0.8 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 164.3, 163.2, 160.3, 142.5, 142.0, 138.9, 135.0, 129.9, 129.8, 129.4, 126.5, 120.6, 116.6, 115.3, 115.1, 114.6, 111.8, 74.1, 69.9, 66.3, 53.3, 50.0, 38.0, 20.7, 13.3; HRMS (ESI) *m/z* calcd for C<sub>25</sub>H<sub>24</sub>FN<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup> 507.1792, found 507.1796; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3432, 2938, 1735, 1654, 1510, 1221, 1018, 821; HPLC: 98.8%, *t*<sub>r</sub> 12.9 min.

**2-((2-((1,2,4)Triazolo[4,3-*b*]pyridazin-6-yloxy)methyl)-2,4-dimethyl-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(3,5-difluorobenzyl)amino)-2-oxoacetic acid (16c).** Pale purple powder, yield 465 mg (49%); mp 129-131 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.40 (s, 3H, 2-CH<sub>3</sub>), 2.85 (s, 3H, *N*-CH<sub>3</sub>), 3.09 (d, *J* 11.9 Hz, 1H, 3-H), 3.35 (d, *J* 11.9 Hz, 1H, 3-H), 4.30 (d, *J* 11.0 Hz, 1H, CH<sub>2</sub>O), 4.35 (d, *J* 10.9 Hz, 1H, CH<sub>2</sub>O), 4.86 (d, *J* 2.9 Hz, 2H, Ph-CH<sub>2</sub>), 6.62-6.67 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.67-6.74 (m, 1H, Ar-H<sup>8</sup>), 6.90 (dd, *J* 8.0, 1.8 Hz, 2H, Ph), 7.03-7.21 (m, 2H, Ph, Ar-H<sup>7</sup>), 8.30 (dd, *J* 9.9, 0.7 Hz, 1H, Ar-H<sup>8</sup>), 9.42 (d, *J* 0.7 Hz, 1H, Ar-H<sup>3</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 164.2, 163.4, 160.3, 142.5, 142.1, 138.9, 135.1, 129.3, 126.6, 120.3, 116.6, 114.3, 112.0, 110.8, 110.6, 102.9, 102.6, 74.1, 69.9, 61.4, 53.2, 50.0, 38.0, 20.6, 13.1; HRMS (ESI) *m/z* calcd for C<sub>25</sub>H<sub>23</sub>F<sub>2</sub>N<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup> 525.1698, found 525.1707; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3432, 2938, 1734, 1654, 1518, 1301, 1119, 824; HPLC: 95.4 %, *t*<sub>r</sub> 13.8 min.

**2-(Benzyl(2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetic acid (17a).** Pale purple powder, yield 493 mg (52%); mp 127-129 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.38 (s, 3H, 2-CH<sub>3</sub>), 2.57 (s, 3H, 3'-CH<sub>3</sub>), 2.84 (s, 3H, *N*-CH<sub>3</sub>), 3.09 (d, *J* 11.9 Hz, 1H, 3-H), 3.32 (d, *J* 11.9 Hz, 1H, 3-H), 4.37 (s, 2H, CH<sub>2</sub>O), 4.80 (d, *J* 4.2 Hz, 2H, Ph-CH<sub>2</sub>), 6.55-6.62 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.66 (d, *J* 8.6 Hz, 1H, Ar-H<sup>8</sup>), 7.05 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.13-7.32 (m, 5H, Ph), 8.21 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 169.8, 168.6, 165.4, 151.5, 148.2, 147.5, 142.0, 140.5, 135.1, 133.8, 133.1, 132.7, 132.1, 125.9, 121.0, 120.0, 117.3, 79.7, 75.1, 64.1, 58.7, 56.2, 43.5, 26.3, 14.7, 9.2; HRMS (ESI) *m/z* calcd for C<sub>26</sub>H<sub>27</sub>N<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup> 503.2043, found 503.2036; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3422, 3063, 2937, 1733, 1655, 1518, 1217, 1020, 819; HPLC: 100%, *t*<sub>r</sub> 13.2 min.

**2-((2,4-Dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)(4-fluorobenzyl)amino)-2-oxoacetic acid (17b).** Pale purple powder,

yield 474 mg (50%); mp 129-131 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.36 (s, 3H, 2-CH<sub>3</sub>), 2.54 (s, 3H, 3'-CH<sub>3</sub>), 2.79 (s, 3H, *N*-CH<sub>3</sub>), 3.00 (d, *J* 11.7 Hz, 1H, 3-H), 3.26 (d, *J* 11.7 Hz, 1H, 3-H), 4.34 (s, 2H, CH<sub>2</sub>O), 4.70 (d, *J* 2.9 Hz, 2H, Ph-CH<sub>2</sub>), 6.25-6.73 (m, 3H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>, Ar-H<sup>8</sup>), 6.97-7.23 (m, 5H, Ph, Ar-H<sup>7</sup>), 8.18 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 162.1, 160.7, 146.8, 142.6, 135.0, 134.4, 130.8, 129.9, 128.6, 127.3, 120.3, 116.2, 115.7, 115.5, 114.6, 112.6, 111.2, 74.9, 70.5, 61.2, 54.3, 50.2, 38.9, 21.7, 13.2, 9.9; HRMS (ESI) *m/z* calcd for C<sub>26</sub>H<sub>26</sub>FN<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup> 521.1949, found 521.1931; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3346, 2936, 1742, 1649, 1503, 1303, 1126, 1022, 820; HPLC: 95.9%, *t*<sub>r</sub> 13.7 min.

**2-((3,5-Difluorobenzyl)(2,4-dimethyl-2-(((3-methyl-[1,2,4]triazolo[4,3-*b*]pyridazin-6-yl)-oxy)methyl)-3,4-dihydro-2*H*-1,4-benzoxazin-7-yl)amino)-2-oxoacetic acid (17c).** Pale purple powder, yield 409 mg (43%); mp 128-130 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 1.39 (s, 3H, 2-CH<sub>3</sub>), 2.56 (s, 3H, 3'-CH<sub>3</sub>), 2.85 (s, 3H, *N*-CH<sub>3</sub>), 3.10 (d, *J* 11.9 Hz, 1H, 3-H), 3.34 (d, *J* 11.9 Hz, 1H, 3-H), 4.37 (s, 2H, CH<sub>2</sub>O), 4.83 (s, 2H, Ph-CH<sub>2</sub>), 6.60-6.65 (m, 2H, Ar-H<sup>5</sup>, Ar-H<sup>6</sup>), 6.69 (d, *J* 8.5 Hz, 1H, Ar-H<sup>8</sup>), 6.86-6.92 (m, 2H, Ph), 7.04 (d, *J* 9.8 Hz, 1H, Ar-H<sup>7</sup>), 7.07-7.15 (m, 1H, Ph), 8.21 (d, *J* 9.8 Hz, 1H, Ar-H<sup>8</sup>); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>): δ 164.1, 163.6, 163.4, 160.0, 142.8, 142.1, 141.3, 135.2, 129.4, 126.7, 120.3, 115.5, 114.6, 112.0, 110.9, 110.6, 102.8, 74.2, 69.7, 61.0, 53.2, 50.0, 38.1, 20.8, 13.1, 9.2; HRMS (ESI) *m/z* calcd for C<sub>26</sub>H<sub>25</sub>F<sub>2</sub>N<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup> 539.1854, found 539.1828; IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$ : 3422, 2937, 2488, 1734, 1656, 1519, 1303, 1118, 822; HPLC: 93.3%, *t*<sub>r</sub> 14.1 min.

### Cell cultures

Bovine aortic endothelial cells (BAEC) were kindly provided by Prof. M. Presta (Brescia, Italy). Human cervical carcinoma (HELA) and human breast carcinoma (MCF-7) cells were obtained from the American Type Culture Collection (ATCC, Middlesex, UK). The cells were grown in Dulbecco's modified minimum essential medium (DMEM, Life Technologies, Inc., Rockville, MD) supplemented with 10 mM Hepes (Life Technologies, Inc., Rockville, MD) and 10% fetal bovine serum (FBS, Harlan Sera-Lab Ltd., Loughborough, UK). Human microvascular endothelial cells (HMEC-1) were obtained from the Centers of Disease Control (CDC, Atlanta, GA) and grown in EGM-2 medium with supplements and growth factors (Lonza, Verviers, Belgium).

### Cell proliferation assays

Cells (HMEC-1, BAEC, HELA or MCF-7) were seeded in 48-well plates at 10,000 cells per cm<sup>2</sup>. After 16 h, the cells were incubated in fresh medium in the presence of the test compounds, as indicated in the Results section. On day 4, (BAEC, HELA, MCF-7) or day 7 (HMEC-1) cells were trypsinized and counted by a Coulter counter (Analis, Belgium).

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