Supporting Information


Paride Liscio, Andrea Carotti, Stefania Asciutti, Tobias Karlberg, Daniele Bellocci, Laura Llacuna, Antonio Macchiariulo, Stuart A Aaronson, Herwig Schüler, Roberto Pellicciari, and Emidio Camaioni.

Table of Contents
Figure 1S  S2
Figure 2S  S3
Computational studies on simplified triazolo[4,3-b]pyridazine nucleus analogs 40-41, 45-46, 49-50.  S4
Figure 3S  S5
Scheme 1S: Synthesis of 8-chloro-6-substituted-[1,2,4]triazolo[4,3-b]pyridazines.  S6
Scheme 2S: Synthesis of 3,6-dichloro-4-pyridazine carboxylic acid  S6
Selectivity data  S7
Table 1S  S7
Figure 4S  S8
Experimental Details  S9
Chemistry  S9
Biology  S20
Computational studies  S21
Crystallography, data collection and structure refinement  S22
Table 2S. Crystal parameters, data collection, and refinement statistics for TNKS-2  S23
Acknowledgments  S24
References  S25
Figure 1S. Superposition of the TNKS-2 protein co-crystallized with 3 (NNL, pdb code 3P0Q, yellow) and 1 (XAV-939, pdb code 3KR8, cyan). These analysis was performed by aligning the backbone atoms of the targets through the Maestro 9.4 “Superposition tool” present in the Schrödinger Suite 2013 (Schrödinger Release 2013-1: Maestro, version 9.4, Schrödinger, LLC, New York, NY, 2013). Hydrogen bond interactions with relevant active site residues are shown in yellow dashes.
Figure 2S. Superposition of the TNKS-2 crystallographic structures in complex with the 3-chloro-4-(4-methyl-2-oxo-1,2-dihydroquinolin-7-y1)benzoic acid (pdb code 4J3M, yellow ligand and green protein) and with compound 12 (ligand cyan and orange protein). These analysis was performed by aligning the backbone atoms of the targets through the Maestro 9.4 “Superposition tool” present in the Schrodinger Suite 2013 (Schrödinger Release 2013-1: Maestro, version 9.4, Schrödinger, LLC, New York, NY, 2013). The main interacting residues defining the binding site near the methyl moiety (Tyr 1050, Tyr1060 and Tyr1071) and the key hydrogen bond interactions with Gly1032 (yellow dashes) are also displayed. The yellow hydrophobic/aromatic surface displayed is generated by the surrounding binding site residues and clearly shows how, in both the ligands, the methyl substituent fits inside it and explain why is crucial for the inhibitory activity.

In an attempt to evaluate the importance of the [1,2,4]triazolo[4,3-b]pyridazine nucleus of the most promising compounds 12, bearing a or p-hydroxyphenylethyl side chain, we replaced it with other different heterocycles (40-41, 45-46, 49-50, Table 1). New derivatives such as [1,2,4]triazolo[1,5-a]pyridines 40 and 41, imidazo[1,2-a]pyridines 45 and 46, and 8- aminoquinolines 49 and 50 were prepared. When analyzed biologically, all of the latter compounds were less potent or inactive as TNKS inhibitors as compared with the parent [1,2,4]triazolo[4,3-b]pyridazine derivatives 32 and 33 (Table 1). To more fully understand the biological results obtained some molecular modeling approaches were applied on TNKS-2 and the most active compounds namely 41, 46 and 50, and compared with their parent analogues [1,2,4]triazolo[4,3-b]pyridazine derivative 33 (Table 1). A protocol of flexible docking performed with the program Glide version 5.92 on the TNKS-2 cocrystal obtained with compound 12, followed by a more accurate MM-GBSA3 rescoring of the best ranked pose performed by Prime version 3.24 was applied. The best scored pose of each compound was analyzed and found to be in line with the observed inhibition activities. The [1,2,4]triazolo[4,3-b]pyridazine derivative 33 (33% of inhibitory activity) displayed the highest number of hydrogen bonds in this series of compounds: two with Gly1032, one with Arg1047 and another with Ser1068 (Figure 3SA). The compound 50 (43% of inhibitory activity) compensated for the loss of an hydrogen bond with Ser1068 with more favorable lipophylic interactions as estimated by the MM-GBSA Lipo PG of binding (-50.45 kcal/mol with respect of -27.86 kcal/mol of 33) with residues Tyr1050, Met1054, Tyr1060 and Tyr1071 (Figure 3SD). The derivatives 41 and 46 (20% and 13% of inhibitory activity, respectively, Figure 3SB and C), showed the loss of an hydrogen bond with Ser1068 if compared with compound 33 together with slightly different geometries of the hydrogen bond formed with the Gly1032, probably less optimal than the ones made by the quinoline 50 and [1,2,4]triazolo[4,3-b]pyridazine derivative 33.
Figure 3S. Binding poses of compounds 33 (A), 41 (B), 46 (C) and 50 (D).
Scheme 1S. Preparation of 8-chloro-6-substituted-[1,2,4]triazolo[4,3-b]pyridazines.\(^a\)

\[\begin{align*}
\text{N} & \text{N} \\
\text{NH}_2 \\
\text{OH} & \\
\text{N} & \\
\text{N} & \\
\text{N} & \\
\text{Cl} & \\
\end{align*}\]

\(51\quad 52, \; R_1 = \text{Me} \quad 54, \; R_1 = \text{Me} \quad 4, \; R_1 = \text{Me} \quad 5, \; R_1 = \text{Et} \)

\(^a\)Reagents and conditions: (a) neat, 160 °C; (b) POCl\(_3\), CH\(_3\)CN, BnEt\(_3\)N\(^+\)Cl\(^-\), reflux.

---

Scheme 2S. Preparation of 3,6-dichloro-4-pyridazine carboxylic acid.\(^a\)

\[\begin{align*}
\text{O} & \\
\text{O} \\
\text{N} & \\
\text{N} & \\
\text{OH} & \\
\text{N} & \\
\text{N} & \\
\text{HO}_2C & \\
\end{align*}\]

\(56\quad 57\quad 58\quad 25\)

\(^a\)Reagents and conditions: (a) NH\(_2\)\(\cdot\)HSO\(_4\), H\(_2\)O, reflux; (b) POCl\(_3\), reflux; (c) KMnO\(_4\), KOH, H\(_2\)O/CH\(_3\)CN-1/1, rt;
Selectivity Data

Compound 12 was profiled at a concentration of 10 µM on several recombinant human PARPs and compared with AZD2281 (Olaparib) and the data are also reported in Table 1S. AZD2281 (olaparib) was employed as positive control for PARP profiling since in our experiments (BPS Bioscience protocols) at 10 µM was able to inhibit all tested PARPs of more than 70% (Table 1S). For comparing, XAV939 IC\textsubscript{50}s are taken from literature. At this concentration 12 selectively inhibited both TNKSs whereas was a weak inhibitor on other PARPs.

**Table 1S.** Selectivity profile of compound 12 in comparison with AZD2281 and XAV939.

<table>
<thead>
<tr>
<th>PARPs</th>
<th>% of inhibition @10 µM (or IC\textsubscript{50}, µM)</th>
<th>Compound 12</th>
<th>AZD 2281</th>
<th>XAV939</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP-1</td>
<td>18</td>
<td>100</td>
<td>(0.0015\textsuperscript{a})</td>
<td>(0.12)\textsuperscript{6}</td>
</tr>
<tr>
<td>PARP-2</td>
<td>26</td>
<td>100</td>
<td>(0.005)\textsuperscript{a}</td>
<td>(0.046)\textsuperscript{6}</td>
</tr>
<tr>
<td>PARP-3</td>
<td>1</td>
<td>100</td>
<td>(0.062)\textsuperscript{a}</td>
<td>(&gt;10)\textsuperscript{6}</td>
</tr>
<tr>
<td>PARP-6</td>
<td>19</td>
<td>100</td>
<td>100</td>
<td>(0.012)</td>
</tr>
<tr>
<td>PARP-7</td>
<td>9</td>
<td>98</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PARP-8</td>
<td>9</td>
<td>89</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PARP-10</td>
<td>4</td>
<td>99</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PARP-11</td>
<td>27</td>
<td>99</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PARP-12</td>
<td>0</td>
<td>71</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>PARP-15</td>
<td>16</td>
<td>83</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are from www.bpsbioscience.com
Figure 4S. Dose-response curve of compound 12 (UPF1854) vs TNKS-1(panel A) and TNKS-2 (panel B).
EXPERIMENTAL DETAILS

$^1$H NMR spectra were recorded at 200 and 400 MHz and $^{13}$C NMR spectra were recorded at 100.6 and 50.3 MHz using the solvents indicated below. Chemical shifts are reported in parts per million (ppm). The abbreviations used are as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; t, triplet; dt, doublet of triplets; qt, quartet of triplets; q, quartet; bs, broad signal. The final products were purified by flash chromatography on silica gel 60 (0.040-0.063 mm). TLC was performed on aluminium backed silica plates (silica gel 60 F254). All the reaction were performed under nitrogen atmosphere using distilled solvent. All reagents were from commercial sources. The LC system was an Agilent 1290 Infinity module equipped with an autosampler, a binary pump, a thermostated column compartment and a diode-array detector. The analytical column was a Zorbax Eclipse Plus (2.1x50mm, 1.8µm). The column temperature was maintained at 40 °C. The mobile phase consisted of an eluent A (water containing 0.1% formic acid) and an eluent B (acetonitrile plus 0.1% formic acid). A 0 min (B= 20%) started a linear gradient at B 80% within 4 min, this mobile phase was maintained for 1 min, at the end of run (5min.) returned back to 20% B. The flow rate was of 0.25 mL/min. The LC system was connected to a detector Agilent 6540 UHD Accurate-Mass Q-TOF/MS system equipped with a source dual Jet Stream. The mass spectrometer operated with positive acquisition, Gas Temp 300°C, gas flow 6.6L/min, nebulizer pressure 16 psi, sheat gas temp 290°C, fragmentor 200V, Skimmer 65V, Octapole RFPeaks 750, Capillary voltage 4000V and Nozzle 0V and Reference masses 121.05087 and 922.009798. The analysis were performed by Mass Hunter workstation. The method EVAL (software Enhanced ChemStation) was used to generate the gradient temperature in the GC-MS analysis on 6850/5975B apparatus (Agilent Technologies, Santa Clara, CA, USA).

Chemistry

Preparation of 8-chloro-6-substituted-[1,2,4]triazolo[4,3-b]pyridazines. Commercially available ethyl $\beta$-ketoesters 52 or 53, (3 mmol) was added of 1-aminotriazole (51) (3 mmol), and stirred at 160 °C (oil bath) for 6 h. After reaction completion the crude was allowed to cooled to rt and ice-water (2 mL) was added to the mixture. The white solid was filtered off, and dried under vacuum thus obtaining the corresponding 6-substituted-[1,2,4]triazolo[4,3-b]pyridazin-8-ols as white powder.

6-Methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-ol (54). White solid; mp >280 °C; $^1$H NMR (200 MHz, DMSO) δ 2.39 (s, 3H), 6.36 (s, 1H), 9.40 (s, 1H). $^{13}$C NMR (100 MHz, DMSO) δ 21.6 102.1, 139.6, 141.0, 152.8, 157.1. Further analytical data are reported elsewhere.7,8

6-Ethyl-[1,2,4]triazolo[4,3-b]pyridazin-8-ol (55). White solid; mp 239-242 °C; $^1$H NMR (400 MHz, DMSO) δ 1.21 (t, 3H, $J = 8$ Hz), 2.71 (q, 2H, $J = 7.5$ Hz), 6.38 (s, 1H), 9.43 (s, 1H); $^{13}$C NMR (100 MHz, DMSO) δ 14.4, 28.0, 100.7, 139.3, 140.7, 152.4, 161.1.

General method B for preparation of 8-chloro-6-substituted-[1,2,4]triazolo[4,3-b]pyridazines. Benzyl triethyl ammonium chloride (16 mmol) was added to a suspension of the corresponding 6-substituted-[1,2,4]triazolo[4,3-b]pyridazin-8-ols (54 or 55) (4 mmol) in dry CH$_3$CN (30 mL). Phosphorous oxychloride (40 mmol) was added at 0 °C to the mixture and stirring was continued at reflux for 16 h. The solvent was removed under vacuum and the excess of phosphorous oxychloride was carefully destroyed by adding cold NaHCO$_3$ (ss) and water. The mixture was extracted with ethyl acetate (4 x 30 mL), the collected organic layers were dried over Na$_2$SO$_4$. 8-chloro-6-substituted-[1,2,4]triazolo[4,3-b]pyridazines were obtained:
8-Chloro-6-methyl-[1,2,4]triazolo[4,3-b]pyridazine (4). Orange solid; mp 188–190 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.60 (s, 3H), 7.11 (s, 1H), 9.0 (s, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.5, 121.3, 129.4, 132.4, 132.8, 154.7; GC-MS \(R_t = 22.36\) min, \(m/z\) 50 (8), 51 (17), 62 (7), 73 (6), 76 (11), 78 (54), 86 (6), 168 (100, M\(^+\)), 169 (8), 170 (32). Further analytical data are reported elsewhere.\(^7\)\(^9\)

8-Chloro-6-ethyl-[1,2,4]triazolo[4,3-b]pyridazine (5). White solid; mp 101-103 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.36 (t, 3H, \(J = 7.5\) Hz), 2.88 (q, 2H, \(J = 7.5\) Hz), 7.11 (s, 1H), 9.0 (s, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 11.6, 28.2, 120.1, 132.3, 139.1, 141.9, 158.7; GC-MS \(R_t = 23.39\) min, \(m/z\), 51 (6), 63 (11), 64 (12), 65 (33), 76 (11), 92 (24), 181 (20), 182 (100, M\(^+\)), 183 (15), 184 (32).

General method C for preparation of N,6-substituted-[1,2,4]triazolo[4,3-b]pyridazin-8-amines. The corresponding arylalkylamines (0.65 mmol) were added to a suspension of anhydrous potassium carbonate (5.4 mmol) and 4 or 5 (0.54 mmol) in dry DMF (5 mL). Stirring was continued at 105 °C for 16 h. The mixture was diluted with water (100 mL), extracted with ethyl acetate (3 × 20 mL), the collected organic layers were dried over Na\(_2\)SO\(_4\). The corresponding N,6-substituted-[1,2,4]triazol[4,3-b]pyridazin-8-amines were obtained in high yield as a pure colored solid after shredding from diethyl ether.

N-(4-Chlorophenethyl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (3). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 4-chlorophenyl ethylamine (0.09 mL, 0.65 mmol) the title compound was obtained in 65% (110.3 mg, 0.38 mmol) yield as white solid; mp 258-260 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.32 (s, 3H), 2.94 (t, 2H, \(J = 7.4\) Hz), 3.54 (br s, 2H), 6.0 (s, 1H), 7.32-7.35 (br s, 4H), 7.85 (br s, 1H, NH, exchanged D\(_2\)O), 9.24 (s, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.7, 33.9, 43.8, 93.0, 128.7, 128.7, 129.9, 132.5, 136.5, 138.7, 139.8, 140.4, 156.6; HRMS \(m/z\) (M+H) calculated for C\(_{14}\)H\(_{13}\)ClN\(_5\): 288.10105, found: 288.10072.

N-Phenethyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (6). Following the general method C and starting from 4 (150 mg, 0.88 mmol) and 2-phenethylamine (0.034 mL, 1.06 mmol) the title compound was obtained in 72% yield (151.5 mg, 0.63 mmol) as white solid; mp 229-231 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.43 (s, 3H), 3.04 (t, 2H, \(J = 7.2\) Hz), 3.70 (q, 2H, \(J = 6.9\) Hz), 5.77 (s, 1H), 6.76 (s, 1H, NH, exchanged D\(_2\)O), 7.23-7.33 (br s, 4H), 8.84 (s, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.9, 34.8, 44.0, 92.5, 126.7, 128.6, 128.6, 128.7, 128.7, 138.1, 139.0, 139.9, 140.7, 156.1; GC-MS \(R_t = 27.540\) min, \(m/z\) 91 (14), 93 (7), 133 (30), 134 (7), 149 (100), 150 (8), 162 (87), 163 (10), 207 (18), 253 (11, M\(^+\)); HRMS \(m/z\) (M+H) calculated for C\(_{14}\)H\(_{16}\)N\(_5\): 254.1400, found: 254.1405.

6-Methyl-N-(2-(naphthalen-2-yl)ethyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (7). Following the general method C and starting from 4 (100 mg, 0.58 mmol) and 2-naphthylethylamine (120 mg, 0.7 mmol) the title compound was obtained in 79% yield (140 mg, 0.46 mmol) as pure yellow solid; mp 233-238 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.39 (s, 3H), 3.19 (t, 2H, \(J = 7.1\) Hz), 3.72 (q, 2H, \(J = 7.0\) Hz), 5.7 (s, 1H), 6.48 (s, 1H, exchanged D\(_2\)O), 7.36 (dd, 1H, \(J_d = 1.6\) Hz, \(J_d = 8.3\) Hz), 7.45-7.48 (m, 2H), 7.69 (s, 1H), 7.76-7.82 (m, 3H), 8.83 (s, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.9, 35.0, 43.9, 92.6, 125.6, 126.2, 126.8, 127.1, 127.4, 127.6, 128.4, 132.3, 133.5, 135.4, 139.0, 139.9, 140.6, 156.1; HRMS \(m/z\) (M+H) calculated for C\(_{18}\)H\(_{18}\)N\(_5\): 304.1556, found: 304.1562.

6-Methyl-N-(2-thiophen-2-yl)ethyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (8). Following the general method C and starting from 4 (50 mg, 0.29 mmol) and thiophene ethylamine (0.034 mL, 0.299 mmol) the title compound was obtained in 86% yield (65 mg, 0.25 mmol) as brownish pure solid; mp 188–194 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.44 (s, 3H), 3.27 (t, 2H, \(J = 6.6\) Hz), 5.80 (s, 1H), 6.59 (s, 1H, NH, exchanged D\(_2\)O), 6.91 (s, 1H), 6.95 (t, 1H, \(J = 3.4\) Hz).
6-Methyl-N-(2-(pyridin-4-yl)ethyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (9). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 2-(2-pyridyl) ethylamine (0.083 mL, 0.7 mmol) the title compound was obtained in 72% yield (108 mg, 0.42 mmol) as white solid; mp 223–227 °C; \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.4, 28.5, 43.5, 92.2, 123.7, 125.2, 126.6, 138.5, 139.4, 139.6, 140.1, 155.7; GC-MS \(R_t = 27.562\) min, \(m/z\) 53 (5), 93 (5), 97 (11), 133 (24), 134 (5), 149 (100), 150 (8), 162 (43), 163 (4), 259 (5, \(M^+\)); HRMS \(m/z\) (M+H) calculated for C\(_{12}\)H\(_{14}\)N\(_5\)S\(_2\): 260.0964, found: 260.0969.

N-(4-Fluorophenethyl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (10). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 4-fluorophenyl ethylamine (0.09 mL, 0.71 mmol) the title compound was obtained in 63% yield (100 mg, 0.37 mmol) as white solid; mp 223–227 °C; \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 22.1, 34.0, 44.6, 92.6, 115.9, 115.9, 130, 130.1, 130.1, 139.9, 140.3, 141.3 156.4, 156.8; GC-MS \(R_t = 7.58\) min, \(m/z\) 51 (18), 66 (15), 78 (22), 79 (16), 93 (77), 106 (64), 133 (26), 162 (100), 207 (65), 254 (10, \(M^+\)); HRMS \(m/z\) (M+H) calculated for C\(_{13}\)H\(_{15}\)N\(_6\): 255.1352, found: 255.1355.

N-(4-Methoxyphenethyl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (11). Following the general method C and starting from 4 (200 mg, 1.19 mmol) and 4-methoxyphenyl ethylamine (0.2 mL, 1.42 mmol) the title compound was obtained in 83% yield (280 mg, 0.98 mmol) as brownish solid; mp 230 °C; \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 22.0, 34.0, 44.4 93.2, 115.7 (\(\delta\) \(J_{CF}\) = 21.2 Hz), 115.7 (\(\delta\) \(J_{CF}\) = 21.2 Hz), 131.5 (\(\delta\) \(J_{CF}\) = 7.8 Hz), 131.5 (\(\delta\) \(J_{CF}\) = 7.8 Hz), 136.2 (\(\delta\) \(J_{CF}\) = 3.1 Hz), 140.0, 140.3, 141.4, 156.6, 161.8 (\(\delta\) \(J_{CF}\) = 240.2 Hz); GC-MS \(R_t = 27.303\) min, \(m/z\) 93 (10), 109 (18), 121 (10), 133 (35), 134 (11), 149 (100), 162 (94), 163 (10), 207 (33), 271 (10, \(M^+\)). HRMS \(m/z\) (M+H) calculated for C\(_{14}\)H\(_{15}\)FN\(_2\): 272.1306, found: 272.1311.

4-(2-(6-Methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-ylamino)ethyl)phenol (12). Boron tribromide (0.08 mL, 0.88 mmol) was added at 0 °C to a suspension of 11 (50 mg, 0.18 mmol) in dry DCM (5 mL). Stirring was continued for 16 h at rt. The solvent was removed under vacuum. The resulting mixture was diluted with ice-water and the excess of BBr\(_3\) was carefully destroyed by adding NaHCO\(_3\) (ss). The acqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na\(_2\)SO\(_4\), and the solvent was evaporated under reduced pressure. The title compound was obtained in 62 % yield (30 mg, 0.11 mmol) as pure white solid; mp 212–214 °C; \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 22.1, 34.0, 44.6, 92.6, 115.9, 115.9, 130, 130.1, 130.1, 139.9, 140.3, 141.3 156.4, 156.8; HRMS \(m/z\) (M+H) calculated for C\(_{14}\)H\(_{16}\)N\(_2\)O: 270.13494, found: 270.13495.
4-(2-(6-Methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-ylamino)ethyl)phenyl benzoate (13). Benzoyl chloride (0.02 mL, 0.18 mmol) was added to a suspension of 12 (50 mg, 0.18 mmol) in dry pyridine (4 mL). Stirring was continued for 24 h at rt. The solvent was removed under vacuum, and the crude was purified by flash chromatography eluting with CHCl₃/MeOH from 0% to 3%. The title compound was obtained in 49% yield (33 mg, 0.08 mmol) as white solid; mp 223–227 °C; ¹H NMR (400 MHz, CDCl₃) δ 2.42 (s, 3H), 3.09 (t, 2H, J = 7.4 Hz), 3.60 (t, 2H, J = 7.2 Hz), 5.78 (s, 1H), 7.15 (d, 2H, J = 8.5 Hz), 7.32 (d, 2H, J = 8.4 Hz), 7.50 (t, 2H, J = 7.9 Hz), 8.0 (d, 1H, J = 7.0 Hz), 8.18 (d, 2H, J = 8.3 Hz), 8.83 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 22.1, 34.5, 44.5, 93.6, 122.3, 128.9, 129.7, 130.1, 130.5, 133.0, 134.0, 136.3, 139.2, 140.3, 141.0, 150.1, 157.1, 165.8. HRMS m/z (M+H) calculated for C₂₁H₂₀N₅O₂: 374.1611, found: 374.1613.

N-(4-Aminophenethyl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (14). Following the general method C and starting from 4 (150 mg, 0.89 mmol) and 4-(2-aminoethyl)benzenamine (0.14 ml, 1.06 mmol). The reaction mixture was purified by flash Chromatography, eluting with DCM/MeOH as gradient from 0 to 4%. The title compound 14 was obtained in 38% yield (90 mg, 0.34 mmol) as yellow solid; mp 218–222 °C; ¹H NMR (400 MHz, DMSO) δ 2.33 (s, 3H), 2.75 (t, 2H, J = 7.8 Hz), 3.44 (br s, 2H), 4.87 (s, 2H, exchanged D₂O), 5.98 (s, 1H), 6.49 (d, 2H, J = 8.2 Hz), 6.93 (d, 2H, J = 8.2 Hz), 7.78 (t, 1H, J = 5.7 Hz, exchanged D₂O), 9.25 (s, 1H); ¹³C NMR (100 MHz, DMSO) δ 21.6, 33.6, 44.2, 92.8, 114.5, 114.5, 126.5, 129.6, 129.6, 139.4, 139.8, 140.8, 147.0, 156.5; HRMS m/z (M+H) calculated for C₁₄H₁₇N₆: 269.1509, found: 269.1513.

N-(2-(4-Methylpiperazin-1-yl)ethyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (15). Following the general method C and starting from 4 (100 mg, 0.6 mmol) and 2-(4-methylpiperazin-1-yl)ethanamine (102.9 mg, 0.72 mmol) the title compound was obtained in 61% yield (99 mg, 0.35 mmol) as white solid after purification by flash chromatography eluting with DCM/MeOH as gradient, from 0 to 10%; mp 170–172 °C; ¹H NMR (400 MHz, DMSO) δ 2.17 (s, 3H), 2.35 (br s, 6H), 2.50 (br s, 5H), 2.58 (t, 2H, J = 6.5 Hz), 3.40 (s, 2H), 6.04 (s, 1H), 7.51 (s, 1H, NH, exchanged D₂O), 9.20 (s, 1H); ¹³C NMR (100 MHz, DMSO) δ 21.3, 39.7 (under DMSO), 45.5, 52.4, 52.4, 54.6, 54.6, 55.5, 92.3, 139.1, 139.4, 140.5, 155.7; HRMS m/z (M+H) calculated for C₁₃H₂₂N₇: 276.1931, found: 276.1932.

6-Methyl-N-(2-morpholino-4-ylethyl)[1,2,4]triazolo[4,3-b]pyridazin-8-amine (16). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 4-(2-aminoethyl)morpholine (0.1 mL, 0.7 mmol), the title compound was obtained in 30% yield (44.5 mg, 0.17 mmol) as white solid; mp 176–178 °C; ¹H NMR (400 MHz, CDCl₃) δ 2.42 (s, 3H), 2.50 (br s, 4H), 2.72 (t, 2H, J = 6.7 Hz), 3.38 (q, 2H, J = 5.1 Hz), 3.71–3.74 (br s, 4H), 5.76 (s, 1H, NH, exchanged D₂O), 8.83 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 21.9, 53.2, 53.2, 55.9, 66.7, 66.7, 92.8, 139.1, 139.9, 140.7, 156.1; HRMS m/z (M+H) calculated for C₁₂H₁₉N₆O: 263.1614, found: 263.1621.

N-Benzyl-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (17). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and benzylamine (0.1 mL, 0.7 mmol) the title compound was obtained in 42% yield (59 mg, 0.24 mmol) as white solid; mp 210–215 °C; ¹H NMR (400 MHz, CDCl₃) δ 2.37 (s, 3H), 4.68 (d, 2H, J = 5.8 Hz), 5.77 (s, 1H), 7.29–7.39 (br s, 5H), 7.57 (s, 1H, NH, exchanged D₂O), 8.83 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 21.9, 38.7, 53.2, 53.2, 55.9, 66.7, 66.7, 92.8, 139.1, 139.9, 140.7, 156.1; GC-MS Rt = 26.432 min, m/z 65 (41), 91 (75), 93 (29), 105 (21), 106 (36), 134 (37), 162 (29), 207 (100), 208 (21), 211 (16), 239 (87, M+); HRMS m/z (M+H) calculated for C₁₃H₁₄N₅O: 240.1243, found: 240.1248.
N-(4-Methoxybenzyl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (18). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 4-methoxyphenyl benzylamine (0.1 mL, 0.7 mmol) the title compound was obtained in 41% yield (59 mg, 0.24 mmol) as white solid; mp 188–192 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 2.38 (s, 3H), 3.78 (s, 3H), 4.57 (d, 2H, $J$ = 5.6 Hz), 5.79 (s, 1H), 6.88 (d, 2H, $J$ = 8.6 Hz), 7.28–7.30 (br s, 3H), 8.82 (s, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 21.9, 46.3, 55.2, 93.4, 114.2, 114.2, 128.3, 128.6, 128.6, 139.0, 140.0, 140.6, 156.8; HRMS m/z (M+H) calculated for C$_{14}$H$_{16}$N$_3$: 266.1400, found: 266.1404.

N-(4-Phenpropyl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (19). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 3-phenyl-1-propylamine (0.08 mL, 0.59 mmol) the title compound was obtained in 68% yield (110 mg, 0.41 mmol) as white solid; mp 159–161 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.92 (quin, 2H, $J$ = 6.8 Hz), 5.89 (s, 1H), 7.17 (t, 1H, $J$ = 2.3 Hz), 5.94 (m, 1H, exchanged D$_2$O), 9.24 (s, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 23.2, 31.3, 34.3, 43.4, 93.8, 127.6, 130.1, 130.2, 130.4, 134.5, 142.4, 143.4, 157.5; HRMS m/z (M+H) calculated for C$_{15}$H$_{18}$N$_5$: 268.155, found: 268.156.

N-(2,3-dihydro-1H-inden-2-yl)-6-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (20). Following the general method C and starting from 4 (100 mg, 0.59 mmol) and 2-indanyl amine (0.1 mL, 0.7 mmol) the title compound was obtained in 41% yield (64.2 mg, 0.24 mmol) as brownish solid; mp 181–183 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.30 (t, 3H, $J$ = 7.6 Hz), 2.70 (q, 2H, $J$ = 7.6 Hz), 3.25 (t, 2H, $J$ = 7.0 Hz), 3.69 (q, 2H, $J$ = 6.88 Hz), 5.79 (s, 1H), 6.80 (br s, 1H, NH, exchanged D$_2$O), 6.88 (dd, 1H, $J_d$ = 0.8 Hz, $J_d$ = 3.3 Hz), 6.91–6.93 (br s, 1H), 7.16 (dd, 1H, $J_d$ = 1.18 Hz, $J_d$ = 5.0 Hz), 8.85 (s, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 22.0, 39.5, 39.5, 53.5, 93.1, 124.8, 124.8, 127.1, 127.1, 139.1, 139.1, 139.9, 139.9, 140.0, 156.1; HRMS m/z (M+H) calculated for C$_{13}$H$_{16}$N$_5$: 266.1400, found: 266.1404.

6-Methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (21).$^2$ Boron tribromide (0.06 mL, 0.63 mmol) was added at 0°C to a suspension of 18 (100 mg, 0.37 mmol) in dry DCM (8 mL). Stirring was continued for 16 h at rt. The solvent was removed under vacuum. The resulting mixture was diluted with ice-water and the excess of BBr$_3$ was carefully destroyed by adding NaHCO$_3$ (ss). The acqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na$_2$SO$_4$, and the solvent was evaporated under reduced pressure. The reaction mixture was then purified by flash chromatography eluting with DCM/MeOH as gradient from 0 to 5%. The title compound (27) was obtained in 42% yield (59 mg, 0.24 mmol) as white solid; mp 210–215 °C; $^1$H NMR (400 MHz, DMSO) δ 2.31 (s, 3H), 3.68 (br s, 2H), 6.0 (s, 1H), 9.34 (s, 1H); $^{13}$C NMR (100 MHz, DMSO) δ 22.0, 96.5, 139.9, 140.5, 142.6, 156.8; HRMS m/z (M+H) calculated for C$_6$H$_8$N$_3$: 150.0774, found: 150.0776.

6-Ethyl-N-(2-(thiophen-2-yl)ethyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (22). Following the general method C and starting from 5 (100 mg, 0.54 mmol) and thiophene ethylamine (0.07 mL, 0.65 mmol) the title compound was obtained in 54 % yield (54 mg, 0.19 mmol) as brownish solid; mp 158–165 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ 1.30 (t, 3H, $J$ = 7.6 Hz), 2.70 (q, 2H, $J$ = 7.6 Hz), 3.25 (t, 2H, $J$ = 7.0 Hz), 3.69 (q, 2H, $J$ = 6.88 Hz), 5.79 (s, 1H), 6.80 (br s, 1H, NH, exchanged D$_2$O), 6.88 (dd, 1H, $J_d$ = 0.8 Hz, $J_d$ = 3.3 Hz), 6.91–6.93 (br s, 1H), 7.16 (dd, 1H, $J_d$ = 1.18 Hz, $J_d$ = 5.0 Hz), 8.85 (s, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 11.2, 27.2, 27.3, 42.3, 90.0, 122.4, 123.8, 125.2, 137.3, 138.2, 138.3, 138.9, 159.2; GC-MS $R_t$ = 28.327 min, m/z 54 (6), 97 (9), 121 (5), 148 (14), 149 (9), 162 (10), 163 (100), 164 (10), 177 (6), 273 (5, M$^+$); HRMS m/z (M+H) calculated for C$_{13}$H$_{16}$N$_5$: 274.1121, found: 274.1125.
6-Ethyl-N-(4-methoxyphenethyl)-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (23). Following the general method C and starting from 5 (110 mg, 0.6 mmol) and 4-methoxy phenylethylamine (0.11 mL, 0.72 mmol) the title compound was obtained in 65% yield (115 mg, 0.39 mmol) as brownish solid; mp 182–184 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.31 (t, 3H, \(J = 7.5\) Hz), 2.69 (q, 2H, \(J = 7.5\) Hz), 2.98 (t, 2H, \(J = 6.9\) Hz), 3.62 (q, 2H, \(J = 7.0\) Hz), 3.78 (s, 3H), 5.77 (s, 1H), 6.83 (m, 3H), 7.15 (d, 2H, \(J = 8.2\) Hz), 8.85 (s, 1H). \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 13.1, 29.2, 34.0, 44.2, 55.2, 91.6, 114.1, 114.1, 129.6, 129.6, 130.1, 139.1, 140.1, 140.9, 158.4, 161.0; GC–MS \(R_t = 35.793\) min, \(m/z\) 77 (6), 91 (5), 119 (5), 121 (27), 134 (3), 148 (6), 163 (100), 164 (10), 176 (8), 297 (3, M\(^+\)); HRMS \(m/z\) (M+H) calculated for C\(_{16}\)H\(_{20}\)N\(_5\)O: 298.1662, found: 298.1667.

4-(2-(6-Ethyl-[1,2,4]triazolo[4,3-b]pyridazin-8-ylamino)ethyl)phenol (24). Boron tribromide (0.08 mL, 0.84 mmol) was added at 0 °C to a suspension of 23 (100 mg, 0.33 mmol,) in dry DCM (5mL). Stirring was continued for 16 h at rt. The solvent was removed under vacuum. The resulting mixture was diluted with ice-water and the excess of BBr\(_3\) was carefully destroyed by adding NaHCO\(_3\) (ss). The aqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na\(_2\)SO\(_4\), and the solvent was evaporated under reduced pressure. The title compound was obtained in 95% yield (88 mg, 0.31 mmol) as pure white solid; mp 231–235 °C; \(^1\)H NMR (400 MHz, DMSO) \(\delta\) 1.22 (t, 3H, \(J = 7.5\) Hz), 2.65 (q, 2H, \(J = 7.5\) Hz), 2.83 (t, 2H, \(J = 6.8\) Hz), 3.51 (br s, 2H), 6.0 (s, 1H), 6.68 (d, 2H, \(J = 8.4\) Hz), 7.01 (d, 2H, \(J = 8.4\) Hz), 7.90 (s, 1H, exchanged D\(_2\)O), 9.4 (s, 1H); \(^13\)C NMR (100 MHz, DMSO) \(\delta\) 12.29, 28.8, 36.7, 47.1, 91.8, 115.5, 115.5, 129.2, 130.1, 130.1, 130.1, 139.5, 139.8, 139.9, 156.1, 157.2; HRMS \(m/z\) (M+H) calculated for C\(_{15}\)H\(_{18}\)N\(_5\)O: 284.1506, found: 284.15085.

4-Methyl-pyridazine-3,6-diol (57). Citraconic anhydride (4 g, 35.7 mmol) was added to a boiling suspension of hydrazine sulphate (4.64 g, 35.7 mmol) in water (12 mL). Stirring was continued at reflux for 6 h. The mixture was cooled to 0 °C, the solid was collected, washed with ice-cold water and dried under vacuum. The title compound 57 was obtained in 90% yield (4.05 g, 32.1 mmol) as a white solid; mp 255–257 °C. \(^1\)H NMR (200 MHz, DMSO) \(\delta\) 1.99 (s, 3H), 6.84 (s, 1H). Further analytical data are reported elsewhere.

3,6-Dichloro-4-methyl-pyridazine (58). Phosphorous oxychloride (14 mL) was added to 57 (4.31 g, 34.2 mmol) and the mixture was heated gently at reflux for 2 h. The volatile were removed under reduced pressure, and the residue was collected and dried under vacuum. The title compound 58 was obtained in 80% yield (4.5 g, 27.6 mmol) as violet crystals; mp 82-84 °C (86 °C lit). \(^1\)H NMR (200 MHz, DMSO) \(\delta\) 2.36 (s, 3H), 8.03 (s, 1H). Further analytical data are reported elsewhere.

3,6-Dichloro-pyridazine-4-carboxylic acid (25). To a suspension of 58 (4.5 g, 27.61 mmol) in CH\(_3\)CN (90 mL), was added a 2M solution of potassium hydroxide (4.65 g, 82.8 mmol). Potassium permanganate (8.73 g, 55.2 mmol) was then added portionwise to the mixture over 30 min. Stirring was continued at rt for 6 h. The volatile were removed under vacuo. The mixture was diluted with water and acidified to pH 1 with 3M HCl. The water phase was extracted with EtOAc (3 x 50 mL). The collected organic phases were washed with brine, dried over Na\(_2\)SO\(_4\) and the solvent was evaporated under reduced pressure. The title compound 25 was obtained in 79% yield (4.12 g, 21.4 mmol) as a white solid; mp 130-133 °C; \(^1\)H NMR (200 MHz, DMSO) \(\delta\) 8.32 (s, 1H). Further analytical data are reported elsewhere.

(3,6-Dichloro-pyridazin-4-yl)-carbamic acid tert-butyl ester (26). Et\(_3\)N (2.11g, 20.8 mmol), DPPA (5.77 g, 20.97mmol) and tert-butanol (26.6 mL) were added in turn to a suspension of 25 (4 g, 20.76 mmol) in dioxane (100 mL). The mixture was heated at 110 °C for 5 h. The reaction was cooled to rt and the solvent was removed under reduced pressure. The crude of reaction was
purified by flash chromatography, eluting with petroleum ether/EtOAc as gradient from 0 to 5% for product. The title compound 26 was obtained in 70% yield (3.85 g, 15.5 mmol) as white solid; mp 81-83 °C; $^1$H NMR (200 MHz, CDCl$_3$) δ 1.56 (s, 9H); 7.41 (1H, s), 8.37 (s, 1H).

3,6-Dichloro-pyridazin-4-ylamine (27). Trifluoroacetic acid (20 mL) was added to a solution of 26 (3.85 g, 14.4 mmol) in DCM. Stirring was continued at rt for 16 h. The volatile were removed under reduced pressure. The crude of reaction was diluted with water, extracted with EtOAc (3 x 50 mL). The organic phase was washed with brine, dried over Na$_2$SO$_4$ and the solvent was removed under vacuo. The title compound 27 was obtained in 68% yield (1.6 g, 9.75 mmol) as yellowish solid; mp 194-196 °C; $^1$H NMR (200 MHz, DMSO) δ 6.81 (s, 1H); 7.24 (2H, br s, exchanged D$_2$O); $^{13}$C NMR (100 MHz, DMSO) δ 108.3, 131.4, 146.0, 154.5. Further analytical data are reported elsewhere.

6-Chloro-3-hydrazino-pyridazin-4-ylamine (28). Hydrazine hydrate 60% (1.43 mL, 27.4 mmol) was added to 27 (300 mg, 1.82 mmol). Stirring was continued at 105-110 °C for 35 min. The reaction was cooled to rt. Crushed ice was added to the mixture. The solid was collected, washed with ice-cold water and dried in vacuo at 40 °C. The title compound 28 was obtained in 77% yield (245 mg, 1.41 mmol) as a brownish solid; mp 185-190 °C; $^1$H NMR (200 MHz, DMSO) δ 2.1 (s, 2H), 6.41 (s, 3H); 7.34 (1H, s). Further analytical data are reported elsewhere.

6-Chloro-[1,2,4]triazolo[4,3-b]pyridazin-8-ylamine (29). A suspension of 28 (360 mg, 2.07 mmol) in formic acid (1 mL) was heated at reflux for 1 h. The mixture was cooled to rt, and diluted with water. The water phase was extracted with EtOAc (3 x 15 mL). The organic phase was washed with brine, dried over Na$_2$SO$_4$ and the solvent was removed under vacuo. The title compound was obtained in 60% yield (198 mg, 1.17 mmol), as a white solid; mp 281-283°C; $^1$H NMR (400 MHz, DMSO) δ 6.10 (d, 1H, J = 5.4 Hz), 7.43 (s, 2H); 8.04 (1H, d, J = 5.4 Hz), 9.35 (s, 1H); $^{13}$C NMR (100 MHz, DMSO) δ 94.4, 139.8, 140.8, 143.0, 147.6. Further analytical data are reported elsewhere.

8-Iodo-[1,2,4]triazolo[4,3-b]pyridazine (31). Isoamyl nitrite (1.3 mL, 9.6 mmol) and diiodomethane (10 mL) were added to a suspension of 30 (290 mg, 2.15 mmol) in CH$_3$CN (43 mL). Stirring was continued at reflux for 1 h. The reaction was cooled to rt. The volatile were removed under high vacuum. The residue was then diluted with water and extracted with DCM (3 x 15 mL). The organic phases were washed with brine, dried over Na$_2$SO$_4$ and evaporated under vacuum. The title compound was obtained in 65% yield (345 mg, 1.4 mmol) as a brownish solid; mp 243-246 °C; $^1$H NMR 400 MHz, DMSO) δ 7.92 (d, 1H, J = 4.4 Hz), 7.43 (s, 2H); 8.2 (1H, d, J = 4.5 Hz), 9.77 (s, 1H); $^{13}$C NMR (100 MHz, DMSO) δ 96.6, 130.8, 140.9, 145.9.

8-Iodo-[1,2,4]triazolo[4,3-b]pyridazin-8-yl-amine (32). Following the general method C and starting from 31 (345 mg, 1.4 mmol) and 4-methoxy phenylethylamine (0.23 g, 1.54 mmol) the title compound was obtained in 56% yield (210 mg, 0.78 mmol) as yellowish solid; mp 181-183 °C; 1H NMR (400 MHz, DMSO) δ 2.87 (t, 2H, J = 7.8 Hz), 3.5 (m, 2H), 3.7 (s,
4-[(1,2,4|Triazolo[4,3-b]pyridazin-8-ylamino)-ethyl]-phenol (33). 1 M solution of boron tribromide (1.9 mL, 1.9 mmol) was added at 0 °C to a suspension of 32 (210 mg, 0.78 mmol,) in dry DCM (21 mL). Stirring was continued for 16 h at rt. The solvent was removed under vacuum. The resulting mixture was diluted with ice-water and the excess of BBr$_3$ was carefully destroyed by adding NaHCO$_3$ (ss). The aqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na$_2$SO$_4$, and the solvent was evaporated under reduced pressure. The title compound was obtained in 50% yield (99 mg, 0.39 mmol) as white solid; mp 237-239 °C; $^1$H NMR (400 MHz, DMSO) δ 2.82 (t, 2H, $J = 7.7$ Hz), 3.49 (m, 2H), 6.08 (d, 1H, $J = 5.6$ Hz), 6.67 (d, 2H, $J = 8.5$ Hz), 7.07 (d, 2H, $J = 8.4$ Hz), 8.0 (t, 1H, $J = 5.7$ Hz), 8.07 (d, 1H, $J = 5.5$ Hz), 9.18 (s, 1H), 9.36 (s, 1H); $^{13}$C NMR (100 MHz, DMSO) δ 33.5, 44.3, 91.3, 115.5, 115.5, 129.4, 130.1, 130.1, 139.9, 140.6, 141.7, 147.6, 156.2; HRMS m/z (M+H) calculated for C$_{13}$H$_{14}$N$_5$O: 256.1193, found: 256.1172.

2-Hydrazino-3-nitropyridine (35). Hydrazine monohydrate (0.94 mL, 12.6 mmol) was added to a solution of 34 (1 g, 6.30 mmol) in methanol. Stirring was continued for 16 h at rt. After solvent removal the residue was partitioned between CHCl$_3$ and H$_2$O. The organic layer was drawn off, dried and evaporated under reduced pressure. The title compound was obtained in 86% yield (840 mg, 5.45 mmol) as orange-brown crystals used then without further purification. Analytical data are in agreement with those reported elsewhere.

8-Nitro-1,2,4-triazolo[4,3-a]pyridine (36). A solution of 35 (800 mg, 5.27 mmol) in formic acid (32 mL) was reacted 2,5 h at 150 °C. The solvent was removed under reduced pressure. The crude of reaction was suspended in water 50 ml, filtered washed with water and dried under reduced pressure. The title compound 36 was obtained in 73% yield (632 mg, 3.85 mmol) as brown solid used then without further purification. Analytical data are in agreement with those reported elsewhere.

8-Nitro-[1,2,4]triazolo[2,3-a]pyridine (37). Derivative 36 (2.0 g, 12.18 mmol) was suspended in a saturated solution of sodium bicarbonate (200 mL). Stirring was continued for 1,5 h at rt. The solid was collected and dried under reduced pressure. 37 was obtained in 80 % yield (1,6 g, 9.75 mmol), as red powder used then without further purification. Analytical data are in agreement with those reported elsewhere.

8-Amino-[1,2,4]triazolo[2,3-a]pyridine (38). To a mixture of 10% Pd/C (20 mg) in ethanol (20 mL), intermediate 47 (200 mg, 1.21 mmol) was added and the reaction contet was stirred for 1.5 h at 20 psi 50 °C under hydrogen atmosphere. The reaction crude was filtered through a short pad of celite. The solvent was removed under reduced pressure. The title compound 38 was obtained in 73% yield (114 mg, 0.85 mmol) as white solid, used then without further purification. Analytical data are in agreement with those reported elsewhere.

N-[(1,2,4]triazolo[1,5-a]pyridin-8-yl]-2-(4-methoxyphenyl)acetamide (39). 4-methoxyphenylacetylchloride (0.34 mL, 2.23 mmol), was added dropwise to a solution of 38 (300 mg, 2.23 mmol) and triethylamine (0.29 mL, 2.23 mmol), in dry DCM. Stirring was continued for 16 h under inert atmosphere at rt. The solvent was removed at reduced pressure. The crude of reaction was taken up with water and extracted with EtOAc (3 x 15 mL). The collected organic layers were washed with brine and dried over Na$_2$SO$_4$. The reaction mixture was then purified by
flash chromatography eluting with PET/EtOAc as gradient from 2 to 20%. The title compound 39 was obtained in 79% yield (500 mg, 0.71 mmol), as white solid; mp 94-98 °C; 1H NMR (400 MHz, CDCl3) δ 3.77 (s, 2H), 3.80 (s, 3H), 6.91 (d, 2H, J = 8.4 Hz), 6.98 (d, 1H, J = 7.1 Hz), 7.28 (d, 2H, J = 8.3 Hz), 8.21 (s, 1H), 8.27(d, 1H, J = 6.7 Hz), 8.48 (d, 1H, J = 7.8 Hz), 8.79 (s, 1H); 13C NMR (100 MHz, CDCl3) δ 41.1, 55.7, 114.9, 114.9, 115.0, 116.1, 123.2, 126.3, 127.7, 130.8, 130.8, 114.5, 152.7, 159.5, 171.1. GC-MS Rf = 26.686 min, m/z 77 (17), 78 (19), 91 (15), 121 (100), 134 (75), 148 (37), 161 (31), 249 (6), 264 (41), 282 (65, M+).

N-(4-methoxyphenethyl)-[1,2,4]triazolo[1,5-a]pyridin-8-amine (40). A solution of 39 (322 mg, 1.14 mmol) in dry DCM (10 mL), was added dropwise at 0 °C to a suspension of LiAlH4 (173 mg, 4.56 mmol), in the same solvent. Stirring was continued for 16 h at reflux. The excess of LiAlH4 was carefully destroyed by adding Na2SO4. Stirring was continued for 15 minutes. The solvent was removed at reduced pressure. The crude of reaction was purified by flash chromatography eluting with DCM/MeOH as gradient from 0 to 4%. The title compound 35 was obtained in 77% yield (250 mg, 0.88 mmol) as green solid; mp 78-81 °C; 1H NMR (400 MHz, CDCl3) δ 2.97 (t, 3H, J = 7.2 Hz), 3.51 (q, 3H, J = 7.2 Hz), 3.80 (s, 3H), 6.38 (d, 1H, J = 7.6 Hz), 6.87 (br s, 2H), 7.18 (d, 2H, J = 6.5 Hz), 7.96 (dd, 1H, Jd = 0.7 Hz, Jd = 6.7 Hz), 8.2 (s, 1H); 13C NMR (100 MHz, CDCl3) δ 34.2, 44.7, 55.2, 101.5, 114.1, 114.1, 115.0, 116.5, 129.5, 129.5, 130.6, 136.8, 143.9, 151.7, 158.3; HRMS m/z (M+H) calculated for: C15H17N4O 269.1397, found: 269.13934.

4-(2-([1,2,4]triazolo[1,5-a]pyridin-8-ylamino)ethyl)phenol (41). Absolute boron tribromide (0.08 mL, 3 mmol) was added at 0°C to a suspension of 40 (235 mg, 83 mmol) in dry DCM (20 mL). Stirring was continued for 16 hrs at rt. The solvent was removed under vacuum. The resulting mixture was diluted with methanol and ice-water and the excess of BBr3 was carefully destroyed by adding NaHCO3 (ss). The aqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na2SO4, and the solvent was evaporated under reduced pressure. The title compound was obtained in 74% yield (120 mg, 0.47 mmol) as pure white solid after shredding by diethyl ether and methanol; mp 156-159 °C; 1H NMR (400 MHz, DMSO) δ 2.81 (t, 2H, J = 7.7 Hz), 6.16 (t, 1H, J = 5.7 Hz, NH, exchanged D2O), 6.49 (d, 1H, J = 7.6 Hz), 6.68 (d, 2H, J = 8.3 Hz), 6.94 (t, 1H, J = 7.3 Hz), 7.0 (d, 2H, J = 8.3 Hz), 8.1 (d, 1H, J = 6.62 Hz), 8.31 (s, 1H) 9.15 (s, 1H); 13C NMR (100 MHz, DMSO) δ 34.3, 45.2, 102.1, 116.0, 116.0, 116.4, 116.7, 130.3, 130.4, 137.7, 144.30, 152.6, 156.6; HRMS m/z (M+H) calculated for: C14H15N4O 255.1240, found: 255.12272.

H-Imidazo[1,2-a]pyridin-8-amine (43). 2,3-Diaminopyridine (854 mg, 7.8 mmol) and NaHCO3 (720 mg, 8.58 mmol), were sequentially added to a 50 % wt/wt acqueous solution of chloroacetaldehyde (0.32 mL, 7.8 mmol). Stirring was continued 16 h at rt. The crude of reaction was diluted with 30 mL of brine, and then extracted with n-butanol (3 x 20 mL). The organic layers were combined, dried over Na2SO4, and concentrated. The crude residue was purified by flash chromatography and eluting with DCM/MeOH as gradient from 0 to 5%. The title compound 53 was obtained in 49% yield (500 mg, 0.48 mmol) as green powder. Analytical data are in agreement with those reported elsewhere.15

N-(H-imidazo[1,2-a]pyridin-8-yl)-2-(4-methoxyphenyl)acetamide (44). 4-Methoxyphenylacetylchloride (0.57 mL, 3.75 mmol), was added dropwise to a solution of 43 (500 mg, 3.75 mmol) and Et3N (0.48 mL, 3.75 mmol), in dry DCM. Stirring was continued for 16 h under inert atmosphere at rt. The solvent was removed at reduced pressure. The crude of reaction was taken up with water and extracted with EtOAc (3 x 15 mL). The collected organic layers were
washed with brine and dried over Na₂SO₄. The title compound 44 was obtained in 85% yield (900 mg, 0.54 mmol), as brown oil used then without further purification; ¹H NMR (400 MHz, CDCl₃) δ 3.78 (s, 2H), 3.83 (s, 3H), 6.78 (d, 2H, J = 8.7 Hz), 7.33 (d, 2H, J = 8.7 Hz), 7.51 (d, 1H, J = 1.2 Hz), 7.56 (d, 1H, J = 1.2 Hz), 7.85 (dd, 1H, J₉ = 1.1 Hz, J₈ = 6.4 Hz), 8.17 (d, 1H, J = 7.6 Hz), 8.81 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 43.8, 55.2, 110.4, 113.1, 113.5, 114.4, 114.4, 120.3, 126.2, 127.4, 130.3, 131.3, 131.7, 139.0, 158.9, 170.5.

N-(4-Methoxyphenethyl)H-imidazo[1,2-a]pyridin-8-amine (45). A solution of 44 (470 mg, 1.67 mmol) in dry DCM (10 mL) was added dropwise at 0°C to a suspension of derivative 44 (0.97 mL, 0.97 mmol) in dry DCM (10 mL). Stirring was continued for 16 h at reflux. The reaction was diluted with EtOAc (3 x 15 mL) and extracted with EtOAc (3 x 15 mL). The collected organic layers were washed with brine and dried over Na₂SO₄. The acqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The reaction mixture was then purified by flash chromatography eluting with PET/EtOAc as gradient from 5 to 25%. The title compound 55 was obtained in 78% yield (350 mg, 1.3 mmol) as green oil. ¹H NMR (400 MHz, CDCl₃) δ 2.97 (t, 2H, J = 7.3 Hz), 3.48 (q, 2H, J = 7.3 Hz), 3.80 (s, 3H), 5.26 (br s, 1H, NH, exchanged D₂O), 6.09 (d, 1H, J = 7.4 Hz), 6.65 (t, 1H, J = 6.9 Hz), 6.87 (d, 2H, J = 8.5 Hz), 7.19 (d, 2H, J = 8.5 Hz), 7.47 (s, 1H), 7.51 (d, 1H, J = 6.6 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 34.5, 44.8, 55.2, 96.6, 113.3, 113.6, 114.0, 114.1, 114.2, 129.6, 129.6, 131.1, 131.1, 131.7, 139.3, 158.2; GC-MS Rᵣ = 26.619 min, m/z 78 (6), 91 (4), 118 (14), 121 (5), 133 (31), 134 (4), 146 (100), 147 (10), 267 (11, M⁺); HRMS m/z (M+H) calculated for: C₁₅H₁₈N₂O 268.1444, found: 268.1418.

4-(2-(H-imidazo[1,2-a]pyridin-8-ylamino)ethyl)phenol (46). 1 M solution of boron tribromide in DCM (0.97 mL, 0.97 mmol) was added dropwise at 0°C to a suspension of derivative 55 (260 mg, 0.97 mmol) in dry DCM (10 mL). Stirring was continued for 16 h at rt and then for 4 h at reflux. The solvent was removed under vacuum. The resulting mixture was diluted with ice-water and the excess of BBr₃ was carefully destroyed by adding NaHCO₃ (ss). The acqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The reaction mixture was then purified by flash chromatography and eluting with DCM/MeOH as gradient from 0 to 5%. The title compound 56 was obtained in 43% yield (100 mg, 0.39 mmol), as greenish solid; mp: 176–181 °C; ¹H NMR (400 MHz, DMSO) δ 2.83 (t, 2H, J = 7.48 Hz), 3.37 (q, 2H, J = 7.01 Hz), 5.77 (t, 1H, J = 5.8 Hz), 6.12 (d, 1H, J = 7.40 Hz), 6.70 (d, 2H, J = 8.2 Hz), 6.69 (br s, 1H), 7.0 (d, 2H, J = 8.3 Hz), 7.39 (s, 1H), 7.79 (d, 1H, J = 6.63 Hz), 7.80 (s, 1H) 9.19 (s, 1H); ¹³C NMR (100 MHz, DMSO) δ 34.5, 45.1, 96.8, 114.3, 114.7, 115.2, 116.0, 116.0, 130.4, 130.4, 130.47, 131.6, 137.7, 139.5, 156.5; HRMS m/z (M+H) calculated for: C₂₃H₂₆N₃O 354.1288, found: 354.1288.

2-(4-Methoxyphenyl)-N-(quinolin-8-yl)acetamide (48). 4-methoxyphenylacetylchloride (100 mg, 0.54 mmol), was added dropwise to a solution of 8-aminoquinoline (47) (78.1 mg, 0.54 mmol) and Et₃N (0.07 mL, 0.54 mmol), in dry DCM (3 x 15 mL). The collected organic layers were washed with brine and dried over Na₂SO₄. The title compound 48 was obtained in 90% yield (150 mg, 0.51 mmol), as yellowish solid; mp 72-76 °C; ¹H NMR (400 MHz, CDCl₃) δ 3.85 (s, 5H), 6.97 (d, 2H, J = 8.5 Hz), 7.38 (d, 2H, J = 8.5 Hz), 7.42 (dd, 1H, J₉ = 4.2 Hz, J₈ = 8.2 Hz), 7.52 (br s, 2H), 8.13 (d, 1H, J = 8.2 Hz), 8.73 (d, 1H, J = 4 Hz), 8.78 (d, 1H, J = 7.3 Hz), 9.92 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 44.4, 55.3, 114.4, 114.4, 116.3, 121.4, 121.5, 126.7, 127.3, 127.8, 130.5, 130.5, 134.4, 136.2, 138.4, 148.1, 158.9, 169.9; GC-MS Rᵣ = 26.629 min, m/z 77 (9), 78 (8), 116 (11), 121 (18), 144 (70), 145 (7), 171 (100), 172 (12), 207 (10), 292 (15, M⁺).
N-(4-Methoxyphenethyl)quinolin-8-amine (49). A solution of 48 (190 mg, 0.65 mmol) in dry DCM (8 mL), was added dropwise at 0 °C to a suspension of LiAlH₄ (98 mg, 2.6 mmol) in the same solvent (20 mL). Stirring was continued for 16 h under inert atmosphere at rt. The reaction was diluted with EtOAc. The excess of LiAlH₄ was carefully destroyed by adding Na.SO₄• 10 H₂O. The mixture was filtrated and the solvent was removed at reduced pressure. The reaction mixture was then purified by flash chromatography eluting with PET/EtOAc as gradient from 5 to 15%. The title compound 59 was obtained in 52% conversion yield (126 mg, 0.45 mmol) as yellowish amorphous solid. ¹H NMR (400 MHz, CDCl₃) δ 3.0 (t, 2H, J = 7.4 Hz), 3.56 (t, 2H, J = 7.2 Hz), 3.81 (s, 3H), 6.26 (s, 1H), 6.71 (d, 1H, J = 7.2 Hz), 6.88 (d, 2H, J = 8.0 Hz), 7.0 (dd, 1H, Jd = 0.8 Hz, Jd = 8.1 Hz), 7.21 (d, 2H, J = 8.7 Hz), 7.36 (dd, 1H, Jd = 4.1 Hz, Jd = 8.3 Hz), 7.39 (t, 1H, J = 8 Hz), 8.0 (dd, 1H, Jd = 1.6 Hz, Jd = 8.2 Hz), 8.7 (dd, 1H, Jd = 1.6 Hz, Jd = 4.2 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 34.6, 45.1, 55.2, 104.6, 113.7, 113.9, 113.9, 121.3, 127.7, 128.6, 129.6, 129.6, 131.5, 135.9, 138.2, 144.5, 146.8, 158.1; GC-MS Rₜ = 26.796 min, m/z 129 (9), 155 (6), 156 (9), 157 (100), 158 (12), 171 (5), 207 (11), 278 (7, M⁺); HRMS m/z (M+H) calculated for: C₁₈H₁₉N₂O₂ 279.1492, found: 279.1127.

4-(2-(Quinolin-8-ylamino)ethyl)phenol (50). Boron tribromide (0.07 mL, 0.78 mmol) was added at 0°C to a suspension of 49 (110 mg, 0.39 mmol) in dry DCM (10 mL). Stirring was continued for 16 h at rt. The solvent was removed under vacuum. The resulting mixture was diluted with iced-water and the excess of BBr₃ was carefully destroyed by adding NaHCO₃ (ss). The aqueous phase was extracted with EtOAc (3 x 15 mL), dried over Na₂SO₄, and the solvent was evaporated under reduced pressure. The reaction mixture was then purified by flash chromatography eluting with PET/EtOAc as gradient from 5 to 30%. The title compound 50 was obtained in 68% yield (70 mg, 0.26 mmol), as yellow solid; mp 143–145 °C; ¹H NMR (400 MHz, DMSO) δ 2.86 (t, 2H, J = 7.3 Hz), 3.40 (q, 2H, J = 6.9 Hz), 6.39 (t, 1H, J = 5.7 Hz, NH, exchanged D₂O), 6.70 (br s, 3H), 7.0 (dd, 1H, Jd = 0.7 Hz, Jd = 8.1 Hz), 7.1 (d, 2H, J = 8.4 Hz), 7.36 (t, 1H, J = 7.8 Hz), 7.46 (dd, 1H, Jd = 4.16 Hz, Jd = 8.2 Hz), 8.1 (dd, 1H, Jd = 1.7 Hz, Jd = 8.2 Hz), 8.6 (dd, 1H, Jd = 1.7 Hz, Jd = 4.1 Hz), 9.2 (s, 1H); ¹³C NMR (100 MHz, DMSO) δ 34.6, 45.2, 105.1, 114.0, 116.0, 116.0, 122.5, 128.7, 129.1, 130.4, 130.4, 130.4, 136.8, 138.3, 145.2, 147.7, 156.5; HRMS m/z (M+H) calculated for: C₁₇H₁ₙN₂O₂ 267.1492, found: 267.13558.
Biology

**PARP assays.** In general, all PARP assays were done by following the BPS PARP assay kit protocols (BPS Bioscience Inc, San Diego, USA) using human recombinant proteins: 80501-PARP1; 80502-PARP2; 80504-TNKS-1; 80515-TNKS-2; 80503-PARP3; 80506-PARP6; 80507-PARP7; 120921-PARP-8; 80510-PARP10; 80511-PARP11; 50513-PARP12. Assay Conditions: the enzymatic reactions for PARP1 and 2 were conducted at least in duplicate at rt for 1 h in a 96 well plate coated with histone substrate. For TNKSs assay, GST-TNKSs was coated on the glutathione plate for the autoribosylation reaction, instead of histone. 50 µl of reaction buffer (Tris•HCl, pH 8.0) contains NAD+, biotinylated NAD+, activated DNA, a PARP enzyme and the test compound. After enzymatic reactions (1 h at RT for PARPs; 1 h at 30 °C for TNKSs), 50µl of Streptavidin-horseradish peroxidase was added to each well and the plate was incubated at rt for an additional 30 min. 100 µl of developer reagents were added to wells and luminescence was measured using a BioTek SynergyTM 2 microplate reader. PARP activity assays were performed at least in duplicates. The luminescence data were analyzed using the computer software, Graphpad Prism.

**Cell culture and growth analysis assay.** DLD-1 colorectal cancer cell line were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (GIBCO, Carlsbad, CA, USA) and cultured at 37 °C in 5% CO₂. For colony growth assay, 5 × 10³ DLD-1 cells were seeded in 60-mm plates. Cells were treated daily with 5, 10, 20 or 40 µM of either compound 12, or XAV939 (1) which were previously dissolved in DMSO at a concentration of 10 mM. At 10 days, cells were washed with phosphate-buffered saline, fixed in 10% methanol/acetic acid solution and stained with 1% crystal violet.

**Quantification of TCF-mediated luciferase reporter activity.** To determine TCF-luciferase reporter activity, cells were transduced with TOP TCF reporter lentivirus-expressing firefly luciferase together with renilla luciferase lentivirus (1:20 ratio) used to normalize for infection efficiency. 24h after infection, cells were lysed and analyzed utilizing the dual luciferase reporter assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Luciferase reporter activity was calculated by dividing TOP/RL ratio.
Computational studies

All the programs used are available in the Schrodinger Suite 2013 (Schrödinger Release 2013-1, LLC, New York, NY, 2013). All the protein structures used in this study were submitted to the “Protein Preparation Wizard” protocol using the default settings to add hydrogens and check the structures. The grid to perform the flexible docking with the program Glide version 5.9 in Single Precision mode (SP) on the TNKS-2 co-crystal, was obtained centering the grid box on the compound 18 and leaving all the other settings by default. A more accurate MM-GBSA rescoring performed by Prime version 3.2 on the best ranked docking pose were applied. All the images and hydrogen bond studies were done using Pymol (The PyMOL Molecular Graphics System, Version 1.6.0 Schrödinger, LLC) and Maestro 9.4 (Schrödinger Release 2013-1: Maestro, version 9.4, Schrödinger, LLC, New York, NY, 2013).
Crystallography, data collection and structure refinement

Protein production and crystallization. The cDNA coding for full-length human TNKS-2 was obtained from Origene (accession code NM_025235). The fragment coding for residues 946-1162 was subcloned into expression vector pNIC-Bsa4, adding an N-terminal hexahistidine tag and a TEV-protease cleavage site (MHHHHHHSSGVDLGTEENLYFQ*SM, where * indicates the site of cleavage). Recombinant expression in *Escherichia coli* and purification of TNKS-2 followed the previously published procedures. Briefly, TNKS-2 was purified from lysed cells using immobilized metal affinity chromatography (IMAC) followed by size exclusion chromatography. Proteins were typically >90% pure as judged by SDS-PAGE analysis. Purified proteins were verified by Time-of-Flight Mass Spectrometry analysis. Crystals of TNKS2 were obtained by sitting drop vapor diffusion at 4 °C in droplets consisting of 0.1 µl protein solution (13.7 mg/ml) and 0.2 µl of well solution (0.1 M Tris-HCl pH 8.0, 19% PEG3350, and 0.2 M ammonium sulfate). Crystals of the TNKS-2–12 complex were obtained by addition of inhibitor directly to drops with crystals, and incubating at 4 °C for 2 days. Soaked crystals were briefly transferred to cryo solution (well solution supplemented with 25% glycerol and 0.2 M NaCl) and stored in liquid nitrogen.

Data collection, structure solution, and refinement. Diffraction data was collected to 1.95Å resolution at the Diamond synchrotron radiation light source, beamline I04 (Oxfordshire, United Kingdom). Data were indexed and integrated in space group C2221 using XDS software. The structure was solved by molecular replacement using the apo structure of human Tankyrase-2 (pdb: 2kr7) as model template and Phaser software. The asymmetric unit contained two protein monomers. The structure was refined using Refmac5 and model building was done using Coot. For further details on data processing and refinement statistics, see Supplementary Table S1. Geometry of the models was analyzed with Molprobity.
Table 2S. Crystal parameters, data collection, and refinement statistics for Tankyrase-2.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Compound 12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
</tr>
<tr>
<td>Xray source</td>
<td>Diamond I04</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97949</td>
</tr>
<tr>
<td>Space group</td>
<td>C2221</td>
</tr>
<tr>
<td>Unit cell dimensions, a, b, c (Å)</td>
<td>91.74, 96.73, 118.61</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30.0 - 1.95 (2.00 – 1.95)</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>36970 (2787)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>8.4 (73.9)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>95.3 (98.1)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>5.1 (5.0)</td>
</tr>
<tr>
<td>&lt;I&gt;/&lt;σI&gt;</td>
<td>15.1 (3.5)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30.0 – 1.95 (2.00 - 1.95)</td>
</tr>
<tr>
<td>Rall (%)</td>
<td>17.76 (26.90)</td>
</tr>
<tr>
<td>Rfree (%)</td>
<td>22.58 (31.40)</td>
</tr>
<tr>
<td>r.m.s.d. bond length (Å)</td>
<td>0.020</td>
</tr>
<tr>
<td>r.m.s.d. bond angle (°)</td>
<td>1.97</td>
</tr>
<tr>
<td><strong>Ramachandran plot</strong></td>
<td></td>
</tr>
<tr>
<td>Most favoured (%)</td>
<td>97.8</td>
</tr>
<tr>
<td>Allowed (%)</td>
<td>100</td>
</tr>
</tbody>
</table>

* Values in parentheses are for the outermost resolution shell.

\[
\text{Rmerge} = \frac{\sum |I - \langle I \rangle|}{\sum I}, \text{ where } I \text{ is the intensity measurement for a given reflection and } \langle I \rangle \text{ is the average intensity for multiple measurements of this reflection.}
\]

\[
R = \frac{\sum |F_{\text{obs}}| - |F_{\text{calc}}|}{\sum |F_{\text{obs}}|}, \text{ where } R_{\text{free}} \text{ is calculated for a randomly chosen 5\% of reflections, which were not used for structure refinement, and } R_{\text{all}} \text{ is calculated for all reflections.}
\]

* The Ramachandran plot was calculated using Molprobity.²⁰
Acknowledgments

The authors thank Dr. Roberto Pellegrino (UNIPG) for technical support on (HPLC-QTOF) HRMS analyses. We wish also to thank the Protein Science Facility at Karolinska Institutet (http://psf.ki.se) and the staff at beamline I04 by the Diamond Light Source for help in crystallography.
References


S26