Structure-based design of dimeric bisbenzimidazole inhibitors to an emergent trimethoprim-resistant type II dihydrofolate reductase guide the design of monomeric analogs

Authors

Jacynthe L. Toulouse[1,2,3], Brahm J. Yachnin[2,4], Edward H. Ruediger[5], Daniel Deon[5], Marc Gagnon[5], Kévin Saint-Jacques[6], Maximilian C. C. J. C. Ebert[7], Delphine Forge[8], Dominic Bastien[1,2,3], Jean Jacques Vanden Eynde[8], Albert M. Berghuis[2,4], Anne Marinier[5] and Joelle N. Pelletier (joelle.pelletier@umontreal.ca)[1,2,3,9]

[1]Département de biochimie et de [9]chimie, Université de Montréal, QC, Canada, [2]PROTEO, the Québec Network for Research on Protein, Function, Engineering and Applications, [3]CGCC, the Center in Green Chemistry and Catalysis, Montréal, QC, Canada, [4]Department of Biochemistry, McGill University, Montréal, QC, Canada, [5]Institute for Research in Immunology and Cancer (IRIC), Université de Montréal, QC, Canada, [6]Département de chimie, Université de Sherbrooke, QC, Canada, [7]Chemical Computing Group, QC, Canada, [8]Laboratoire de chimie organique, Université de Mons, Belgium.

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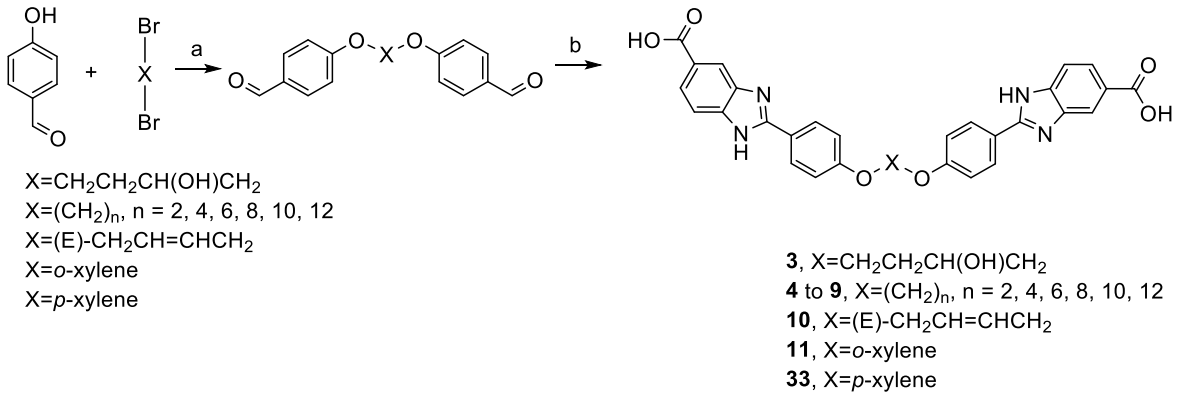
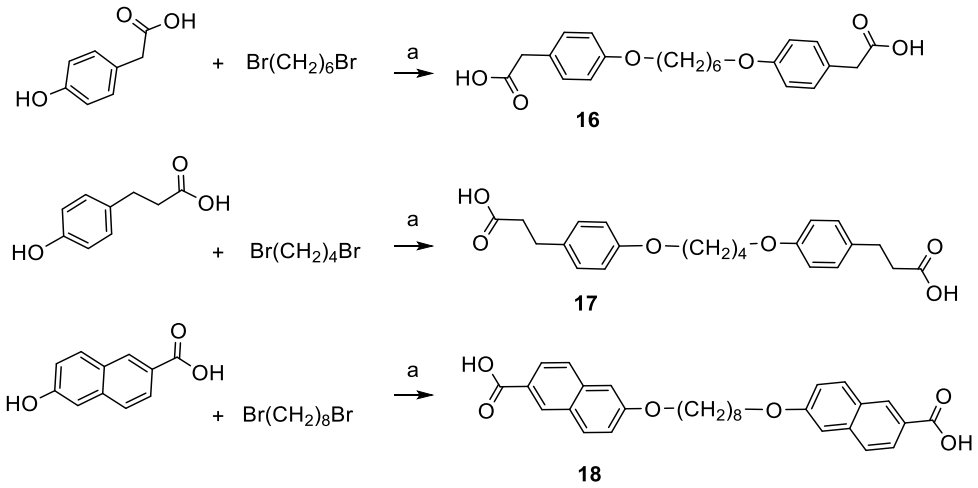
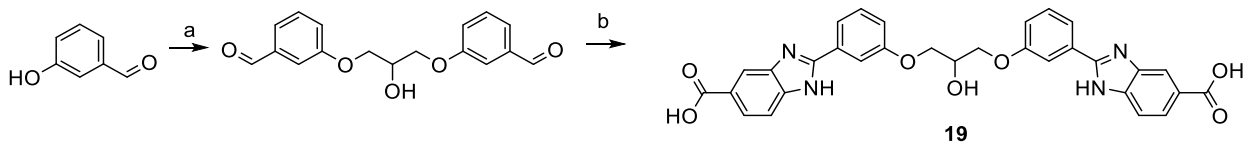
Supplementary synthetic schemes.



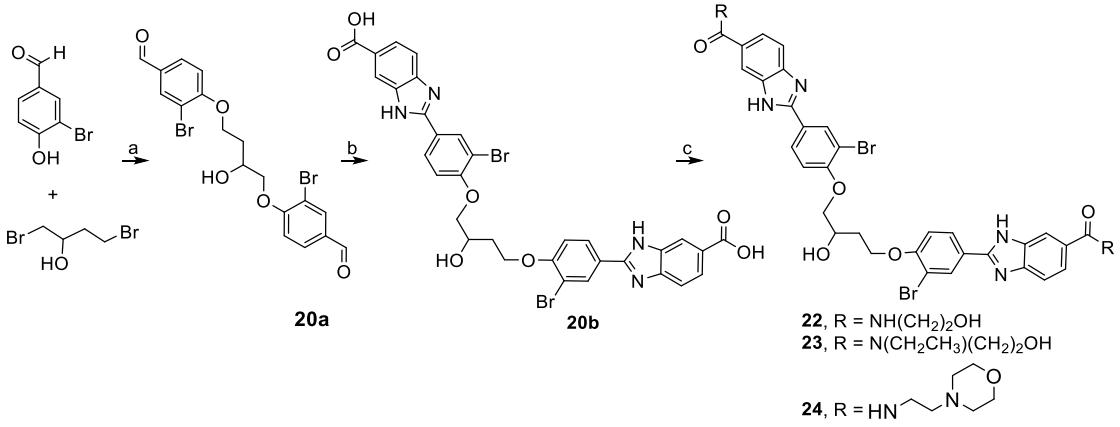
Scheme S1. Synthesis of **3** to **11** and **33**. Reagents and conditions: a) EtOH/H_2O (9:1), NaOH, 120°C, 20 min. b) $Na_2S_2O_5$, 3,4-diaminobenzoic acid, EtOH/H_2O (3:1), 140°C, 15 min, 19-43%.



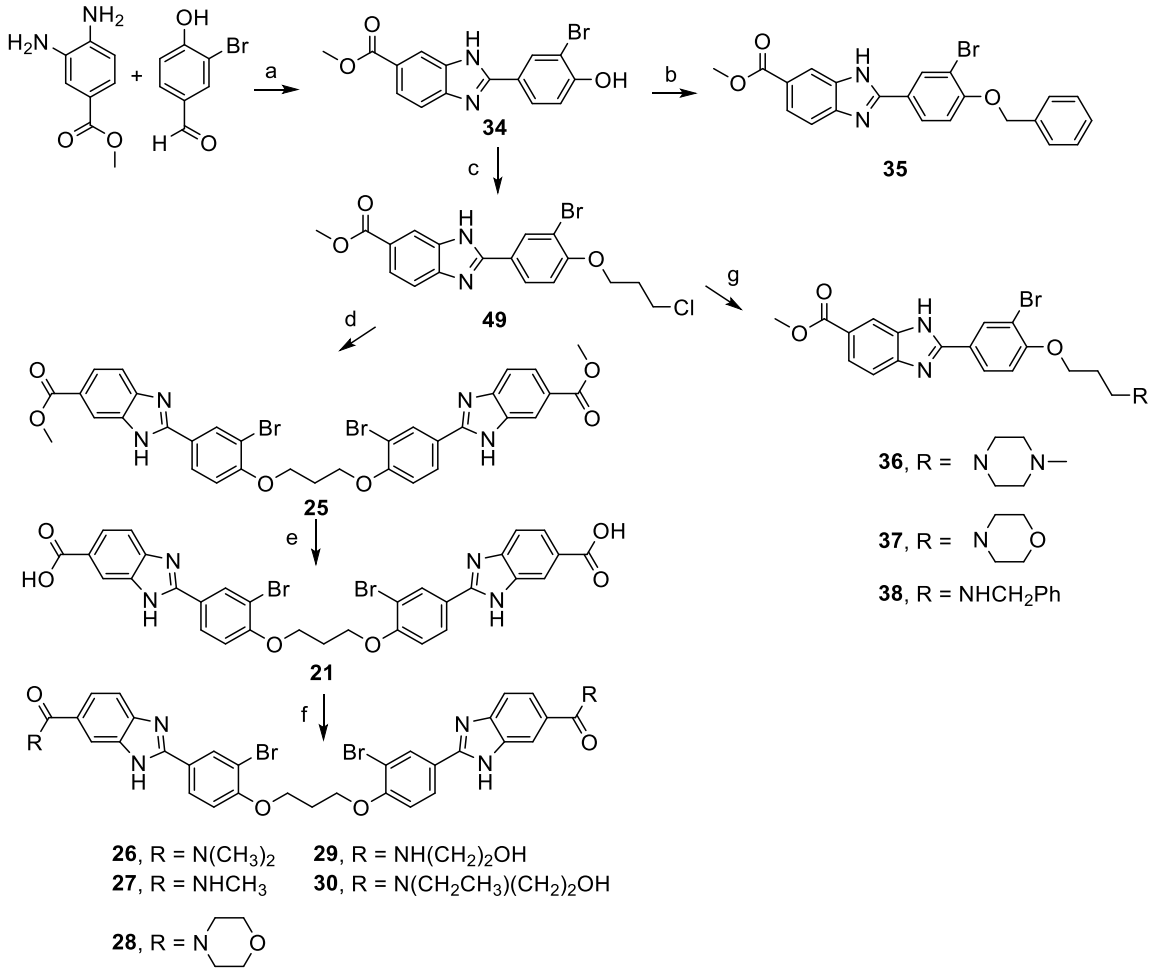
Scheme S2. Synthesis of **16** to **18**. Reagents and conditions: a) EtOH/H_2O (9:1), NaOH, 120°C, 20 min, 8-10%.



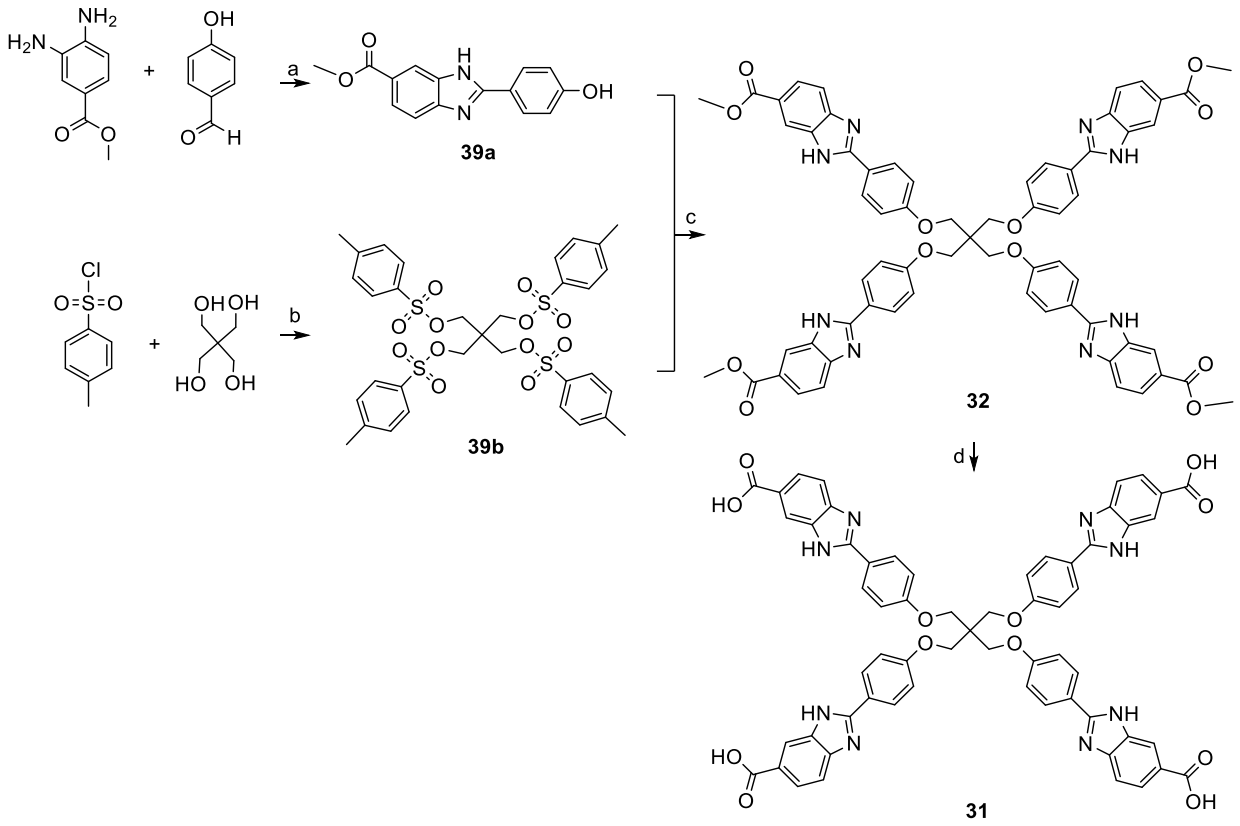
Scheme S3. Synthesis of **19**. Reagents and conditions: a) 1,3-dibromopropan-2-ol, EtOH/H_2O (9:1), NaOH, 120°C, 20 min. b) $Na_2S_2O_5$, 3,4-diaminobenzoic acid, EtOH/H_2O (3:1), 140°C, 15 min.



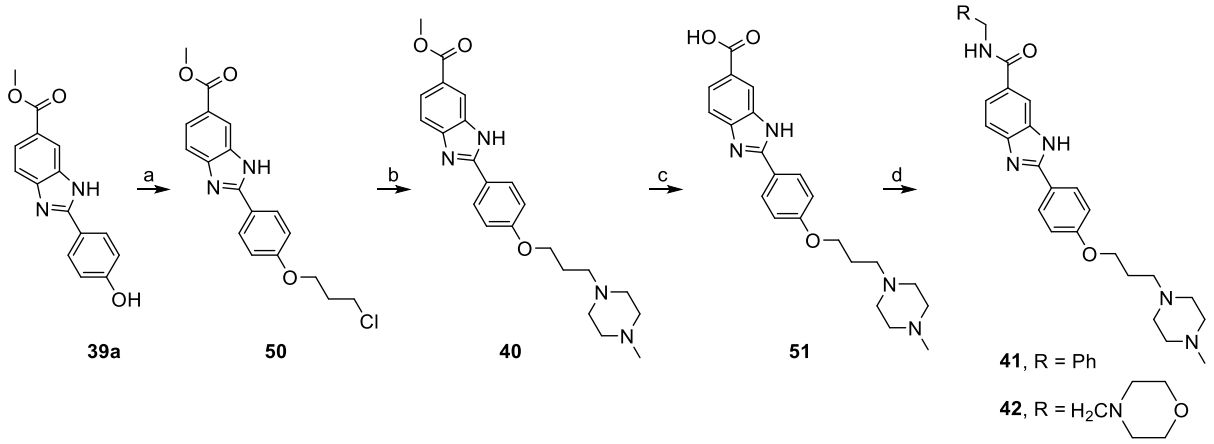
Scheme S4. Synthesis of brominated **20b** and its derivatives. Reagents and conditions: a) EtOH, K_2CO_3, reflux, 8h, 39% b) $Na_2S_2O_5$, 3,4-diaminobenzoic acid, EtOH/H_2O, 140°C, 15 min, 84% c) ethanolamine for **22**, 2-(ethylamino)ethanol for **23** or 2-morpholinoethanamine for **24**, HATU, DIPEA, DMF, rt, 3h, 50-92%.



Scheme S5. Synthesis of 2-(3-bromophenyl)benzimidazole-6-carboxylate **21** and its derivatives. Reagents and conditions: a) DMF, $Na_2S_2O_5$, 80°C, 3h, 70%. b) Cs_2CO_3, acetonitrile, benzyl bromide, 70°C, 16h, 9%. c) Cs_2CO_3, acetone, 1-bromo-3-chloropropane, 70°C, 16h, 27%. d) **35**, NaI, DMF, 90°C, 22h, 26-44%. e) NaOH/MeOH/THF (1:1:3), 70°C, 1h, 72%. f) dimethylamine for **26**, methylamine for **27**, morpholine for **28**, ethanolamine for **29** or 2-(ethylamino)ethanol for **30**, HATU, DIPEA, DMF, rt, 3h, 42-56%. g) 1-methylpiperazine for **36**, morpholine for **37** or benzylamine for **38**, Cs_2CO_3, NaI, DMF, 100°C, 20h, 17%.

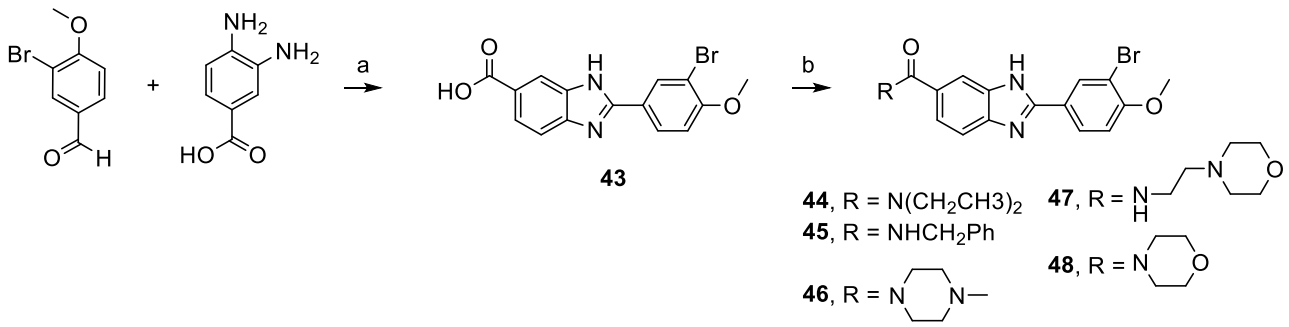


Scheme S6. Synthesis of **31**, **32** and **39a-b**. Reagents and conditions: a) $Na_2S_2O_5$, DMF, 80°C, 3h, 69%. b) pyridine, rt, 48h, 64%. c) DMF, K_2CO_3, reflux, 24h, 23%. d) 1M NaOH/MeOH/THF (1:1:3), 70°C, 1h, 42%.



Scheme S7. Synthesis of non-symmetrical phenylbenzimidazole-6-carboxylate compounds. Reagents and conditions: a) Cs_2CO_3, acetone, 1-bromo-3-chloropropane, 80°C, 16h, 33%. b) 1-

methylpiperazine, Cs_2CO_3, NaI, DMF, 100°C, 20h, 56% c) 1M NaOH/MeOH/THF (1:1:3), 70°C, 1h. d) benzylamine for **41** or 2-morpholinoethanamine for **42**, HATU, DIPEA, DMF, rt, 3h, 36%.



Scheme S8. Synthesis of non-symmetrical 2-phenylbenzimidazole compounds. Reagents and conditions: a) anhydrous DMF, $Na_2S_2O_5$, 80°C, 3h. b) diethylamine hydrochloride for **44**, benzylamine for **45** or 1-methylpiperazine for **46**, 2-morpholinoethanamine for **47** or morpholine for **48**, HATU, DIPEA, DMF, rt, 3h, 45-63%.

Supplementary synthetic methods.

2,2'-(((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(4,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (3). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,4-dibromo-2-butanol (1.73 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid (0.91 g, 2.9 mmol, 19%). ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.08 (s, 2H), 12.49 (br, 1H), 8.20 (br, 1H), 8.15 (m, 4H), 8.10 (br, 1H), 7.84 (d, J = 5.8 Hz, 2H), 7.66 (br, 1H), 7.56 (br, 1H), 7.16 (m, 4H), 5.02 (s, 1H), 4.29 (t, J = 6.6 Hz, 2H), 4.11 (m, 3H), 2.12 (m, 1H), 1.95 (m, 1H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 161.05, 160.97, 154.7 (br), 153.8 (br), 147.8 (br), 144.1 (br), 138.8 (br), 135.0 (br), 128.9, 124.9, 124.0 (br), 123.5 (br), 122.8, 122.7, 120.7 (br), 118.5 (br), 115.6, 115.5, 113.2 (br), 111.2 (br), 73.0, 66.2, 65.2, 33.7. Purity > 95%.

2,2'-((Ethane-1,2-diylbis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (4). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,2-dibromoethane (1.40 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified

product was a white solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.92 (br), 8.17 (m, 4H), 8.14 (br, 2H), 7.83 (d, *J* = 8.4 Hz, 2H), 7.62 (br, 1H), 7.59 (br, 1H), 7.21 (m, 4H), 4.50 (s, 4H). ^{13}C-NMR (176 MHz, DMSO) δ = 168.4, 160.6, 154.0, 128.9, 125.6 (br), 125.4 (br), 123.8, 123.2, 115.6, 67.2. Purity 93%.

2,2'-((Butane-1,4-diylbis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (5). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,4-dibromobutane (1.60 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.89 (s, 2H), 12.44 (br, 2H), 8.21 (br, 1H), 8.14 (d, *J* = 8.1 Hz, 4H), 8.10 (br, 1H), 7.82 (d, *J* = 5.0 Hz, 2H), 7.66 (br, 1H), 7.56 (br, 1H), 7.15 (d, *J* = 8.1 Hz, 4H), 4.19 (t, *J* = 5.4 Hz, 4H), 1.97 (t, *J* = 5.4 Hz, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 161.0, 154.7 (br), 153.8 (br), 147.8 (br), 144.1 (br), 138.9 (br), 135.2 (br), 128.9, 124.9, 124.0 (br), 123.6 (br), 122.7, 120.7 (br), 118.5 (br), 115.6, 113.2 (br), 111.2 (br), 68.1, 25.9. Purity > 95%.

2,2'-((Hexane-1,6-diylbis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (6). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,6-dibromohexane (1.82 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.89 (br, 2H), 12.38 (br, 2H), 8.35 (br, 2H), 8.13 (d, *J* = 8.3 Hz, 4H), 7.82 (d, *J* = 8.0 Hz, 2H), 7.60 (br, 2H), 7.13 (d, *J* = 8.3 Hz, 4H), 4.12 (t, *J* = 6.3 Hz, 4H), 1.82 (m, 4H), 1.56 (t, *J* = 6.9 Hz, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 161.0, 154.5 (br), 153.9 (br), 128.9, 125.0, 123.8 (br), 122.6, 115.5, 68.3, 29.1, 25.7. Purity > 95%.

2,2'-((Octane-1,8-diylbis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (7). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,8-dibromooctane (2.03 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a yellow solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.07 (br, 1H), 13.04 (br, 1H), 12.68 (br, 2H), 8.20 (br, 1H), 8.13 (m, 4H), 8.09 (br, 1H), 7.83 (br, 2H), 7.66 (br, 1H), 7.55 (br, 1H), 7.12 (m, 4H), 4.10 (t, *J* = 6.5 Hz, 4H), 1.78 (m, 4H), 1.48 (m, 4H), 1.41 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 161.0, 154.7 (br), 153.8 (br), 147.8 (br), 144.1 (br), 138.8 (br), 135.2 (br), 128.9, 124.9, 124.0 (br), 123.4 (br), 122.6, 120.7 (br), 118.5 (br), 115.5, 113.2 (br), 111.2 (br), 68.4, 29.13, 29.10, 25.9. Purity > 95%.

2,2'-((Decane-1,10-diylbis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (8). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,10-dibromodecane (2.24 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.06 (br, 1H), 13.02 (br, 1H), 12.64 (br, 2H), 8.21 (br, 1H), 8.12 (m, 4H), 8.09 (br, 1H), 7.82 (br, 2H), 7.66 (br, 1H), 7.56 (br, 1H), 7.11 (m, 4H), 4.08 (t, *J* = 6.5 Hz, 4H), 1.77 (m, 4H), 1.45 (m, 4H), 1.38 (m, 4H), 1.34 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 161.0, 154.7 (br), 153.8 (br), 147.8 (br), 144.2 (br), 138.9 (br), 135.2 (br), 128.9, 124.9, 124.0 (br), 123.5 (br), 122.6, 120.7 (br), 118.5 (br), 115.5, 113.2 (br), 111.2 (br), 68.4, 29.3, 29.2, 29.1, 25.9. Purity > 95%.

2,2'-((Dodecane-1,12-diylbis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (9). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,10-dibromododecane (2.45 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.00 (br, 1H), 12.68 (br, 2H), 8.16 (br, 2H), 8.12 (m, 4H), 7.82 (dd, *J* = 1.4, 8.3 Hz, 2H), 7.62 (br, 1H), 7.59 (br, 1H), 7.11 (m, 4H), 4.07 (t, *J* = 6.5 Hz, 4H), 1.75 (m, 4H), 1.45 (m, 4H), 1.36 (m, 4H), 1.31 (m, 8H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 161.0, 154.2, 128.9, 124.9, 123.7 (br), 122.6, 115.5, 68.3, 29.39, 29.37, 29.2, 29.1, 25.9. Purity > 95%.

(E)-2,2'-((But-2-ene-1,4-diylbis(oxy))bis(4,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (10). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and *trans*-1,4-dibromo-2-butene (1.60 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid (1.55 g, 5.3 mmol, 35%). ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.17 (br, 2H), 8.19 (br, 1H), 8.14 (m, 4H), 8.09 (br, 1H), 7.83 (d, *J* = 5.9 Hz, 2H), 7.65 (br, 1H), 7.59 (br, 1H), 7.17 (m, 4H), 6.15 (t, *J* = 2.7 Hz, 2H), 4.74 (d, *J* = 2.7 Hz, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.4, 160.4, 154.7 (br), 153.9 (br), 147.8 (br), 144.2 (br), 138.6 (br), 135.2 (br), 128.9 (2C), 124.7, 124.1 (br), 123.4 (br), 122.7, 120.8 (br), 118.6 (br), 115.6, 113.4 (br), 111.3 (br), 67.9. Purity > 94%.

2,2'-(((1,4-Phenylenebis(methylene))bis(oxy))bis(4,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (11). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,4-bis(bromomethyl)benzene (1.97 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a light brown solid (1.92 g, 5.6 mmol, 37%). ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.91 (br, 2H), 12.18 (br, 2H), 8.18 (br, 2H), 8.15 (m,

4H), 7.82 (d, *J* = 8.3 Hz, 2H), 7.61 (br, 2H), 7.54 (s, 4H), 7.23 (m, 4H), 5.25 (s, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 160.6, 137.0, 128.9, 128.3, 124.9, 123.9 (br), 123.0, 115.9, 69.8. Purity > 94%.

4,4'-(Hexane-1,6-diylbis(oxy))dibenzoic acid (**12**). Synthesis of **12** was done as described previously.[1] ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.59 (br, 2H), 7.87 (m, 4H), 7.01 (m, 4H), 4.05 (t, *J* = 6.5 Hz, 4H), 1.76 (m, 4H), 1.49 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 167.5, 162.8, 131.8, 123.3, 114.7, 68.2, 29.0, 25.7. Purity = 97%

4,4'-(Octane-1,8-diylbis(oxy))dibenzoic acid (**13**). Synthesis of **13** was done as described previously.[1] ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.59 (br, 2H), 7.88 (m, 4H), 7.00 (m, 4H), 4.04 (t, *J* = 6.5 Hz, 4H), 1.73 (m, 4H), 1.43 (m, 4H), 1.36 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 167.5, 162.8, 131.8, 123.2, 114.7, 68.2, 29.1, 29.0, 25.9. Purity = 93%

4,4'-(Decane-1,10-diylbis(oxy))dibenzoic acid (**14**). Synthesis of **14** was done as described previously.[1] ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.37 (br, 2H), 7.88 (m, 4H), 7.00 (m, 4H), 4.05 (t, *J* = 6.4 Hz, 4H), 1.73 (m, 4H), 1.43 (m, 4H), 1.35 (m, 4H), 1.30 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 167.4, 162.8, 131.8, 123.5, 114.8, 68.4, 29.3, 29.1, 29.0, 25.9. Purity > 95%.

4,4'-(Dodecane-1,12-diylbis(oxy))dibenzoic acid (**15**). Synthesis of **15** was done as described previously.[1] ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.60 (br, 2H), 7.88 (m, 4H), 6.99 (m, 4H), 4.05 (t, *J* = 6.5 Hz, 4H), 1.73 (m, 4H), 1.42 (m, 4H), 1.34 (m, 4H), 1.29 (m, 8H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 167.4, 162.8, 131.8, 123.5, 114.8, 68.4, 29.35, 29.32, 29.1, 29.0, 25.9. Purity > 95%.

2,2'-((Hexane-1,6-diylbis(oxy))bis(4,1-phenylene))diacetic acid (**16**). Synthesis of **16** was done as described previously.[2] Briefly, 4-hydroxyphenylacetic acid (0.76 g, 5 mmol) and 1,6-dibromohexan-2-ol (0.65 g, 2.5 mmol) were reacted according to General procedure C. The purified product was a white solid (0.17 g, 0.45 mmol, 9%). ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.24 (br, 2H), 7.15 (m, 4H), 6.86 (m, 4H), 3.94 (t, *J* = 6.4 Hz, 4H), 3.48 (s, 4H), 1.73 (m, 4H), 1.47 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 173.5, 157.9, 130.8, 127.3, 114.7, 67.8, 40.3, 29.1, 25.8. Purity > 95%.

3,3'-((Butane-1,4-diylbis(oxy))bis(4,1-phenylene))dipropionic acid (**17**)**.** Synthesis of **17** was done as described previously.[2] Briefly, 3-(4-hydroxyphenyl)propanoic acid (0.83 g, 5 mmol) and 1,4-dibromobutan-2-ol (0.58 g, 2.5 mmol) were reacted according to General procedure C. The purified product was a white solid (0.15 g, 0.4 mmol, 8%). ^{1}H-NMR (700 MHz, DMSO) δ ppm

12.08 (br, 2H), 7.12 (m, 4H), 6.84 (m, 4H), 3.98 (m, 4H), 2.75 (t, *J* = 7.6 Hz, 4H), 2.48 (t, *J* = 7.6 Hz, 4H), 1.84 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 174.3, 157.4, 133.2, 129.7, 114.8, 67.5, 36.1, 30.0, 26.0. Purity ≈ 82%.

6,6'-((Octane-1,8-diylbis(oxy))bis(4,1-phenylene))bis(2-naphthoic acid) (18). Synthesis of **18** was done as described previously.[2] Briefly, 6-hydroxy-2-naphthoic acid (0.94 g, 5 mmol) and 1,8-dibromooctan-2-ol (0.72 g, 2.5 mmol) were reacted according to General procedure C. The purified product was a white solid (0.34 g, 0.5 mmol, 10%). ^{1}H-NMR (700 MHz, DMSO) δ ppm 12.87 (br, 2H), 8.51 (s, 2H), 8.01 (d, *J* = 9.0 Hz, 2H), 7.92 (d, *J* = 8.4 Hz, 2H), 7.87 (d, *J* = 8.4 Hz, 2H), 7.40 (s, 2H), 7.23 (d, *J* = 8.4 Hz, 2H), 4.13 (t, *J* = 6.2 Hz, 4H), 1.81 (m, 4H), 1.49 (m, 4H), 1.41 (m, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.0, 159.0, 137.2, 131.3, 130.8, 127.9, 127.3, 126.2, 126.1, 120.1, 107.1, 68.2, 29.2, 29.0, 26.0. Purity > 95%.

2,2'-(((2-Hydroxypropane-1,3-diyl)bis(oxy))bis(3,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (19). 3-hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,3-dibromopropan-2-ol (1.62 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a grey solid. ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.01 (br, 2H), 8.20 (br, 2H), 7.85 (m, 4H), 7.80 (d, *J* = 7.9 Hz, 2H), 7.65 (br, 2H), 7.49 (dd, *J* = 7.9, 7.9 Hz, 2H), 7.17 (d, *J* = 7.9 Hz, 2H), 4.30 (m, 3H), 4.24 (m, 2H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.5, 159.7, 131.6, 130.6, 119.8, 117.5, 113.3, 70.3, 68.2; 7. Purity = 90%.

4,4'-((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromobenzaldehyde) (20a). A solution of 3-bromo-4-hydroxybenzaldehyde (1.820 g, 9.06 mmol), 1,4-dibromobutan-2-ol (1.00 g, 4.31 mmol) and potassium carbonate (0.596 g, 4.31 mmol) in ethanol (11 mL) was stirred while being heated to reflux for 8 h. The cooled mixture was filtered, and the filter-cake was washed with water, ethanol and diethyl ether and then dried in vacuo to give the title compound as a white solid (0.800 g, 1.69 mmol, 39%). The product obtained was used as such in the next step without further characterization.

2,2'-(((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[*d*]imidazole-6-carboxylic acid) (20b). A mixture of 4,4'-((2-hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromobenzaldehyde) (**20a**) (0.15 g, 0.318 mmol), 3,4-diaminobenzoic acid (0.102 g, 0.667 mmol), sodium metabisulfite (0.060 g, 0.318 mmol), water (0.315 mL, 17.47 mmol) and ethanol (0.835 mL, 14.30 mmol) was heated at 140°C in a microwave reactor for 15 min. The cooled mixture was filtered, and the filter-cake was washed with water, ethanol and

diethyl ether and then dried in vacuo to give the title compound as a white solid (0.196 g, 0.266 mmol, 84%). LC (Analytical HPLC- General method A): 1.890 min. HRMS (ESI): calcd for $C_{32}H_{25}Br_2N_4O_7$ $[M+H]^+$ *m/z* 735.0085, found 735.0077. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 13.15 (br s, 2H), 8.40 (t, *J* = 1.76 Hz, 2H), 8.02 - 8.26 (m, 4H), 7.83 (d, *J* = 8.61 Hz, 2H), 7.50 - 7.74 (m, 1H), 7.30 - 7.41 (m, 1H), 5.21 (br s, 1H), 4.28 - 4.43 (m, 2H), 4.10 - 4.25 (m, 3H), 2.13 - 2.28 (m, 1H), 1.99 (dt, *J* = 4.70, 8.41 Hz, 1H). Purity = 88%.

2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (21). A mixture of dimethyl 2,2'-((propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylate) (**25**) (0.100 g, 0.136 mmol), 1 M aqueous NaOH (2.18 mL, 2.18 mmol), MeOH (2.18 mL) and THF (6.54 mL) was heated at 70°C for 1 h. The cooled mixture was then concentrated to dryness and the residue was treated with a 1 M HCl solution. The solid present was collected by filtration, washed with water and dried under high vacuum to give the title compound as a white solid (0.069 g, 0.098 mmol, 72% yield). LC (Analytical HPLC- General method B): 1.329 min. HRMS (ESI): calcd for $C_{31}H_{23}Br_2N_4O_6$ $[M+H]^+$ *m/z* 706.9961, found 706.9967. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.92 (br s , 2h), 8.39 - 8.53 (m, 2H), 8.09 - 8.33 (m, 4H), 7.90 (dd, *J* = 1.57, 8.61 Hz, 2H), 7.70 (d, *J* = 8.61 Hz, 2H), 7.45 (d, *J* = 8.61 Hz, 2H), 4.32 - 4.53 (m, 4H), 2.34 (dd, *J* = 5.48, 6.26 Hz, 3H). Purity = 85%.

2,2'-(((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(N-(2-hydroxyethyl)-1H-benzo[d]imidazole-6-carboxamide) (22). 2,2'-(((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**20b**) (0.025 g, 0.034 mmol) and ethanolamine (4.52 µl, 0.075 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.016 g, 0.019 mmol, 57%). LC (Analytical HPLC- General method A): 1.720 min. HRMS (ESI): calcd for $C_{36}H_{35}Br_2N_6O_7$ $[M+H]^+$ *m/z* 821.0928, found 821.0925. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.49 (t, *J* = 5.48 Hz, 2H), 8.42 (t, *J* = 1.76 Hz, 2H), 8.19 (td, *J* = 2.74, 8.61 Hz, 2H), 8.13 (s, 2H), 7.82 (d, *J* = 8.61 Hz, 2H), 7.65 (d, *J* = 8.61 Hz, 2H), 7.40 (dd, *J* = 3.72, 8.80 Hz, 2H), 4.33 - 4.42 (m, 2H), 4.13 - 4.25 (m, 3H), 3.51 - 3.58 (m, 4H), 3.37 (q, *J* = 6.26 Hz, 4H), 2.17 - 2.26 (m, 1H), 1.95 - 2.05 (m, 1H). Purity > 99%.

2,2'-(((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(N-ethyl-N-(2-hydroxyethyl)-1H-benzo[d]imidazole-6-carboxamide) (23). 2,2'-(((2-Hydroxybutane-1,4-

diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**20b**) (0.025 g, 0.034 mmol) and 2-(ethylamino)ethanol (7.24 µl, 0.075 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.015 g, 0.017 mmol, 50%). LC (Analytical HPLC- General method A): 1.773 min. HRMS (ESI): calcd for $C_{40}H_{43}Br_2N_6O_7$ $[M+H]^+$ *m/z* 877.1554, found 877.1562. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (t, *J* = 1.96 Hz, 2H), 8.18 (td, *J* = 2.54, 8.61 Hz, 2H), 7.58 - 7.69 (m, 4H), 7.40 (dd, *J* = 3.72, 8.80 Hz, 2H), 7.29 (d, *J* = 8.61 Hz, 2H), 4.32 - 4.42 (m, 2H), 4.14 - 4.26 (m, 3H), 3.34 (br s, 12H), 2.14 - 2.29 (m, 1H), 1.90 - 2.07 (m, 1H), 1.14 (br s, 6H). Purity > 99%.

((((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-2,6-diyl))bis(morpholinomethanone) (24). 2,2'-(((2-Hydroxybutane-1,4-diyl)bis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**20b**) (0.025 g, 0.034 mmol) and 2-morpholinoethanamine (9.80 µl, 0.075 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.030 g, 0.031 mmol, 92%). LC (Analytical HPLC- General method A): 1.646 min. HRMS (ESI): calcd for $C_{44}H_{49}Br_2N_8O_7$ $[M+H]^+$ *m/z* 959.2085, found 959.2094. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.75 (t, *J* = 5.67 Hz, 2H), 8.41 (t, *J* = 1.96 Hz, 2H), 8.19 (td, *J* = 2.74, 8.61 Hz, 2H), 8.14 (s, 2H), 7.79 (d, *J* = 9.39 Hz, 2H), 7.66 (d, *J* = 8.61 Hz, 2H), 7.38 (dd, *J* = 2.93, 8.80 Hz, 2H), 4.36 (t, *J* = 6.65 Hz, 2H), 4.12 - 4.25 (m, 3H), 3.95 - 4.09 (m, 4H), 3.53 - 3.74 (m, 12H), 3.30 - 3.41 (m, 4H), 3.08 - 3.24 (m, 4H), 2.16 - 2.28 (m, 1H), 1.93 - 2.07 (m, 1H). Purity > 99%.

Dimethyl 2,2'-((propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[*d*]imidazole-6-carboxylate) (25). A vial was charged with methyl 2-(3-bromo-4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**49**) (0.030 g, 0.071 mmol), potassium carbonate (0.020 g, 0.142 mmol), sodium iodide (10.6 mg, 0.071 mmol), methyl 2-(3-bromo-4-hydroxyphenyl)-1H-benzo[d]imidazole-6-carboxylate (0.025 g, 0.071 mmol) and DMF (1.5 mL). The resulting mixture was heated at 90°C for 22 h and then the cooled mixture was diluted with chloroform and the solids were removed by filtration. The filtrate was then concentrated to dryness and the resulting crude residue was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as an amorphous white solid (0.009 g, 0.012 mmol, 17% yield). LC (Analytical HPLC- General method A): 2.156 min. HRMS (ESI): calcd for

$C_{33}H_{27}Br_2N_4O_6$ $[M+H]^+$ *m/z* 735.0275, found 735.0290. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (d, *J* = 1.96 Hz, 2H), 8.18 (dd, *J* = 2.35, 8.61 Hz, 2H), 8.13 - 8.16 (m, 2H), 7.86 (dd, *J* = 1.76, 8.41 Hz, 2H), 7.67 (d, *J* = 8.22 Hz, 2H), 7.41 (d, *J* = 9.00 Hz, 2H), 4.41 (t, *J* = 6.06 Hz, 4H), 3.88 (s, 6H), 2.33 (td, *J* = 5.77, 11.93 Hz, 2H). Purity = 89%.

2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(N,N-dimethyl-1H-benzo[d]imidazole-6-carboxamide) (26). 2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**21**) (0.015 g, 0.021 mmol) and dimethylamine (2.367 µl, 0.047 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.007 g, 9.20 µmol, 43%). LC (Analytical HPLC- General method A): 1.903 min. HRMS (ESI): calcd for $C_{35}H_{33}Br_2N_6O_4$ $[M+H]^+$ *m/z* 759.0925, found 759.0917. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.43 (d, *J* = 2.35 Hz, 2H), 8.19 (dd, *J* = 2.35, 8.61 Hz, 2H), 7.64 (dd, *J* = 3.33, 4.89 Hz, 4H), 7.43 (d, *J* = 8.61 Hz, 2H), 7.27 - 7.33 (m, 2H), 4.42 (t, *J* = 6.26 Hz, 4H), 2.99 (br s, 12H), 2.29 - 2.40 (m, 2H). Purity > 99%.

2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(N-methyl-1H-benzo[d]imidazole-6-carboxamide) (27). 2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**21**) (0.015 g, 0.021 mmol) and methylamine (1.45 mg, 0.047 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.007 g, 9.56 µmol, 45%). LC (Analytical HPLC- General method A): 1.895 min. HRMS (ESI): calcd for $C_{33}H_{29}Br_2N_6O_4$ $[M+H]^+$ *m/z* 731.0612, found 731.0590. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.46 (q, *J* = 4.30 Hz, 2H), 8.43 (d, *J* = 2.35 Hz, 2H), 8.20 (dd, *J* = 2.35, 8.61 Hz, 2H), 8.09 (s, 2H), 7.77 (dd, *J* = 1.57, 8.61 Hz, 2H), 7.63 (d, *J* = 8.61 Hz, 2H), 7.42 (d, *J* = 8.61 Hz, 2H), 4.42 (t, *J* = 6.06 Hz, 4H), 2.82 (d, *J* = 4.70 Hz, 6H), 2.29 - 2.38 (m, 2H). Purity = 88%.

(((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-2,6-diyl))bis(morpholinomethanone) (28). 2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**21**) (0.015 g, 0.021 mmol) and morpholine (4.07 µl, 0.047 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.009 g, 10.66 µmol, 50%). LC (Analytical HPLC- General

method A): 1.866 min. HRMS (ESI): calcd for $C_{39}H_{37}Br_2N_6O_6$ $[M+H]^+$ *m/z* 843.1136, found 843.1126. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.43 (d, *J* = 2.35 Hz, 2H), 8.19 (dd, *J* = 2.35, 8.61 Hz, 2H), 7.59 - 7.70 (m, 4H), 7.43 (d, *J* = 9.00 Hz, 2H), 7.32 (dd, *J* = 1.17, 8.22 Hz, 2H), 4.34 - 4.47 (m, 4H), 3.50 - 3.56 (m, 16H), 2.28 - 2.40 (m, 2H). Purity > 99%.

2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(N-(2-hydroxyethyl)-1H-benzo[d]imidazole-6-carboxamide) (29). 2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**21**) (0.015 g, 0.021 mmol) and ethanolamine (2.8 µl, 0.047 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the desired product as a white solid (0.007 g, 8.83 µmol, 42%). LC (Analytical HPLC- General method A): 1.830 min. HRMS (ESI): calcd for $C_{35}H_{33}Br_2N_6O_6$ $[M+H]^+$ *m/z* 793.0806, found 793.0808. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.46 (t, *J* = 5.67 Hz, 2H), 8.42 (d, *J* = 2.35 Hz, 2H), 8.20 (dd, *J* = 2.35, 8.61 Hz, 2H), 8.11 (s, 2H), 7.74 - 7.81 (m, 2H), 7.63 (d, *J* = 8.61 Hz, 2H), 7.42 (d, *J* = 9.00 Hz, 2H), 4.42 (t, *J* = 6.26 Hz, 4H), 3.54 (t, *J* = 6.26 Hz, 4H), 3.31 - 3.41 (m, 4H), 2.26 - 2.38 (m, 2H). Purity >99%.

2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(N-ethyl-N-(2-hydroxyethyl)-1H-benzo[d]imidazole-6-carboxamide) (30). 2,2'-((Propane-1,3-diylbis(oxy))bis(3-bromo-4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (**21**) (0.015 g, 0.021 mmol) and 2-(ethylamino)ethanol (4.5 µl, 0.047 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.010 g, 0.012 mmol, 56%). LC (Analytical HPLC- General method A): 1.856 min. HRMS (ESI): calcd for $C_{39}H_{41}Br_2N_6O_6$ $[M+H]^+$ *m/z* 847.1449, found 847.1451. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (d, *J* = 2.35 Hz, 2H), 8.19 (dd, *J* = 2.35, 8.61 Hz, 2H), 7.61 (d, *J* = 8.22 Hz, 2H), 7.41 (d, *J* = 9.00 Hz, 2H), 7.23 (d, *J* = 7.83 Hz, 2H), 6.83 (t, *J* = 9.39 Hz, 2H), 4.41 (t, *J* = 6.06 Hz, 2H), 4.15 - 4.29 (m, 4H), 2.83 - 3.13 (m, 4H), 1.99 - 2.15 (m, 4H), 1.80 - 1.96 (m, 6H), 0.74 - 0.89 (m, 6H). Purity = 88%.

2,2'-(((2,2-Bis((4-(6-carboxy-1H-benzo[d]imidazol-2-yl)phenoxy)methyl)propane-1,3-diyl)bis(oxy))bis(4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylic acid) (31). A solution of dimethyl 2,2'-(((2,2-bis((4-(6-(methoxycarbonyl)-1H-benzo[d]imidazol-2-yl)phenoxy)methyl)propane-1,3-diyl)bis(oxy))bis(4,1-phenylene))bis(1H-benzo[d]imidazole-6-carboxylate) (**32**) (0.020 g, 0.018 mmol), 1 M NaOH (0.211 mL, 0.211 mmol), methanol (0.21

mL) and THF (0.63 mL) was stirred at 70°C for 1 h. The cooled mixture was concentrated to dryness, the residue was acidified with 1M aqueous HCl and the mixture was again concentrated to dryness. The residue was then suspended in methanol, collected by filtration and washed with ether to give the title compound as a white solid (0.008 g, 7.40 µmol, 42%). LC (Analytical HPLC-General method A): 1.899 min. HRMS (ESI): calcd for $C_{61}H_{45}N_8O_{12}$ $[M+H]^+$ *m/z* 1081.3151, found 1081.3145. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.98 (br s, 4H), 8.27 (d, *J* = 9.00 Hz, 8H), 8.15 (s, 4H), 7.86 - 7.94 (m, 4H), 7.69 (d, *J* = 8.61 Hz, 4H), 7.31 (d, *J* = 9.00 Hz, 8H), 4.55 (br s, 8H). Purity > 99%.

2,2'-(((2,2-Bis((4-(6-methylcarboxy-1H-benzo[d]imidazol-2-yl)phenoxy)methyl)propane-1,3-diyl)bis(oxy))bis(4,1-phenylene))bis(6-methylcarboxy-1H-benzo[d]imidazole) (32). A mixture of methyl 2-(4-hydroxyphenyl)-1H-benzo[*d*]imidazole-6-carboxylate (**39a**) (0.100 g, 0.373 mmol), 2,2-bis((tosyloxy)methyl)propane-1,3-diyl bis(4-methylbenzenesulfonate) (**39b**) (0.070 g, 0.093 mmol) and potassium carbonate (0.052 g, 0.373 mmol) in DMF (1.75 mL) was stirred while heating to reflux for 24 h. The cooled mixture was concentrated to dryness, the residue was suspended in water and the solid obtained was collected by filtration. The crude solid was then taken up in DMF and purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as an amorphous white solid (0.024 g, 0.021 mmol, 23%). LC (Analytical HPLC-General method A): 2.135 min. HRMS (ESI): calcd for $C_{65}H_{53}N_8O_{12}$ $[M+H]^+$ *m/z* 1137.3777, found 1137.3598. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.05 - 8.16 (m, 12H) 7.82 (dd, *J* = 8.4, 1.8 Hz, 4H) 7.62 (d, *J* = 8.6 Hz, 4H) 7.26 (d, *J* = 9.0 Hz, 8H) 4.53 (br s, 8H) 3.87 (s, 12H). Purity = 82%.

2,2'-(((1,2-Phenylenebis(methylene))bis(oxy))bis(4,1-phenylene))bis(1H-benzo[d]imidazole-5-carboxylic acid) (33). 4-Hydroxybenzaldehyde (1.83 g, 15 mmol) and 1,3-bis(bromomethyl)benzene (1.97 g, 7.5 mmol) were reacted according to General procedure A followed by General procedure B. The purified product was a brown solid (0.79 g, 6.5 mmol, 43%). ^{1}H-NMR (700 MHz, DMSO) δ ppm 13.16 (br, 2H), 8.15 (m, 4H), 8.12 (br, 2H), 7.81 (d, *J* = 8.5 Hz, 2H), 7.63 (br, 2H), 7.60 (m, 2H), 7.43 (m, 2H), 7.26 (m, 4H), 5.38 (s, 4H). ^{13}C-NMR (176 MHz, DMSO) δ ppm 168.3, 160.6, 154.6 (br), 153.7 (br), 135.5, 129.2, 128.9, 128.7, 125.0, 124.0 (br), 123.5 (br), 123.1, 120.8 (br), 118.5 (br), 115.9, 68.0. Purity > 95%.

Methyl 2-(3-bromo-4-hydroxyphenyl)-1H-benzo[d]imidazole-6-carboxylate (**34**). A 100 mL sealed tube was charged with methyl 3,4-diaminobenzoate (1.901 g, 11.44 mmol), 3-bromo-4-

hydroxybenzaldehyde (2.3 g, 11.44 mmol) and DMF (48.7 mL). Sodium metabisulfite (2.175 g, 11.44 mmol) was then added and the resulting mixture was heated at 80°C for 3 h. After cooling, the reaction mixture was concentrated to dryness and the solid residue obtained was suspended in water-acetonitrile (1:1) and sonicated for 30 min. The resulting suspension was filtered, and the residue was dried in vacuo to give the title compound (3.097 g, 8.03 mmol, 70% yield) as a yellow solid. LC (Analytical HPLC- General method A): 1.686 min. HRMS (ESI): calcd for $C_{15}H_{12}BrN_2O_3$ $[M+H]^+$ *m/z* 347.0026, found 347.0035. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.01 (br s, 1H), 8.34 (d, *J* = 2.35 Hz, 1H), 8.15 (d, *J* = 1.57 Hz, 1H), 8.04 (dd, *J* = 2.15, 8.41 Hz, 1H), 7.85 (dd, *J* = 1.76, 8.41 Hz, 1H), 7.65 (d, *J* = 8.22 Hz, 1H), 7.13 (d, *J* = 8.61 Hz, 1H), 3.88 (s, 3H). Purity > 99%.

Methyl 2-(4-(benzyloxy)-3-bromophenyl)-1H-benzo[d]imidazole-6-carboxylate (**35**). To a mixture of methyl 2-(3-bromo-4-hydroxyphenyl)-1H-benzo[d]imidazole-6-carboxylate (**34**) (0.100 g, 0.288 mmol) and cesium carbonate (0.141 g, 0.432 mmol) in acetonitrile (1.35 mL) was added benzyl bromide (0.038 mL, 0.317 mmol) and the resulting mixture was heated at 70°C for 16 h. The cooled mixture was then diluted with chloroform (15 mL) and the insolubles were removed by filtration. The filtrate was concentrated under reduced pressure and the crude residue was taken up in DMF and purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.011 g, 0.025 mmol, 9%). LC (Analytical HPLC- General method A): 2.144 min. HRMS (ESI): calcd for $C_{22}H_{18}BrN_2O_3$ $[M+H]^+$ *m/z* 437.0495, found 437.0522. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.44 (d, *J* = 2.35 Hz, 1H), 8.15 - 8.22 (m, 2H), 7.87 (dd, *J* = 1.76, 8.41 Hz, 1H), 7.68 (d, *J* = 8.22 Hz, 1H), 7.48 - 7.54 (m, 2H), 7.40 - 7.47 (m, 3H), 7.34 - 7.39 (m, 1H), 5.33 (s, 2H), 3.88 (s, 3H). Purity = 92%.

Methyl 2-(3-bromo-4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**36**)**.** Methyl 2-(3-Bromo-4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**49**) (0.020 g, 0.047 mmol) and 1-methylpiperazine (0.014 g, 0.142 mmol) were reacted as follows: a vial was charged with **49** (1.0 eq), cesium carbonate (3.0 eq), sodium iodide (1.0 eq), 1-methylpiperazine (0.014 g, 0.142 mmol, 3.0 eq) and DMF (0.5 mL). The resulting mixture was heated at 100°C for 20 h. The cooled mixture was filtered, and the filtrate was directly purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.010 g, 0.021 mmol, 44%). LC (Analytical HPLC- General method A): 1.547 min. HRMS (ESI): calcd for $C_{23}H_{28}BrN_4O_3$ $[M+H]^+$ *m/z* 487.1339, found 487.1368. ^{1}H NMR (400

MHz, Methanol-d_4) δ ppm 8.35 (d, *J* = 1.17 Hz, 1H), 8.39 (d, *J* = 2.35 Hz, 1H), 8.06 - 8.16 (m, 3H), 7.75 (d, *J* = 8.61 Hz, 1H), 7.33 (d, *J* = 8.61 Hz, 1H), 4.30 (t, *J* = 5.87 Hz, 2H), 3.97 (s, 3H), 3.21 - 3.29 (m, 4H), 2.87 - 3.07 (m, 6H), 2.85 (s, 3H), 2.10 - 2.21 (m, 2H). Purity > 99%.

Methyl 2-(3-bromo-4-(3-morpholinopropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (37). Methyl 2-(3-Bromo-4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**49**) (0.030 g, 0.071 mmol) and morpholine (0.019 g, 0.212 mmol) were reacted as follows: a vial was charged with **49** (1.0 eq), cesium carbonate (3.0 eq), sodium iodide (1.0 eq), morpholine (0.019 g, 0.212 mmol, 3.0 eq) and DMF (0.5 mL). The resulting mixture was heated at 100°C for 20 h. The cooled mixture was filtered, and the filtrate was directly purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.009 g, 0.019 mmol, 27%). LC (Analytical HPLC- General method A): 1.495 min. HRMS (ESI): calcd for $C_{22}H_{25}BrN_3O_4$ $[M+H]^+$ *m/z* 476.1006, found 476.1034. ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm 8.39 (d, *J* = 2.35 Hz, 1H), 8.31 - 8.35 (m, 1H), 8.13 (dd, *J* = 2.35, 8.61 Hz, 1H), 8.08 (dd, *J* = 1.57, 8.61 Hz, 1H), 7.73 (d, *J* = 8.61 Hz, 1H), 7.33 (d, *J* = 8.61 Hz, 1H), 4.34 (t, *J* = 5.67 Hz, 2H), 4.02 - 4.20 (m, 2H), 3.96 (s, 3H), 3.70 - 3.91 (m, 2H), 3.53 - 3.70 (m, 2H), 3.41 - 3.52 (m, 2H), 3.15 - 3.29 (m, 2H), 2.31 - 2.44 (m, 2H). Purity = 98%.

Methyl 2-(4-(3-(benzylamino)propoxy)-3-bromophenyl)-1H-benzo[d]imidazole-6-carboxylate (38). Methyl 2-(3-Bromo-4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**49**) (0.030 g, 0.071 mmol) and benzylamine (0.023 mL, 0.212 mmol) were reacted as follows: a vial was charged with **49** (1.0 eq), cesium carbonate (3.0 eq), sodium iodide (1.0 eq), benzylamine (0.023 mL, 0.212 mmol, 3.0 eq) and DMF (0.5 mL). The resulting mixture was heated at 100°C for 20 h. The cooled mixture was filtered, and the filtrate was directly purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.009 g, 0.018 mmol, 26%). LC (Analytical HPLC- General method A): 1.749 min. HRMS (ESI): calcd for $C_{25}H_{25}BrN_3O_3$ $[M+H]^+$ *m/z* 496.1057, found 496.1085. ^{1}H NMR (400 MHz, Methanol-d_4) δ ppm 8.36 (d, *J* = 2.35 Hz, 1H), 8.30 - 8.34 (m, 1H), 8.12 (dd, *J* = 2.35, 8.61 Hz, 1H), 8.04 (dd, *J* = 1.57, 8.61 Hz, 1H), 7.70 (d, *J* = 8.61 Hz, 1H), 7.45 - 7.56 (m, 5H), 7.26 - 7.32 (m, 2H), 4.33 (t, *J* = 5.67 Hz, 2H), 4.29 (s, 2H), 3.95 (s, 3H), 3.38 (t, *J* = 7.43 Hz, 2H), 2.26 - 2.37 (m, 2H). Purity = 91%.

Methyl 2-(4-hydroxyphenyl)-1H-benzo[d]imidazole-6-carboxylate (39a). A 100 mL sealed tube was charged with methyl 3,4-diaminobenzoate (1.857 g, 11.18 mmol), 4-

hydroxybenzaldehyde (1.365 g, 11.18 mmol), DMF (47.6 mL) and sodium metabisulfite (2.125 g, 11.18 mmol). The resulting reaction mixture was stirred for 3 h at 80°C. The cooled mixture was filtered and the filter-cake was suspended in water-acetonitrile (1:1) and sonicated for 30 min. The resulting slurry was filtered and the obtained solid was dried in vacuo to give the title compound as a yellow solid (2.057 g, 7.67 mmol, 69%). Purification of a small portion of this material (60 mg) by prep HPLC (Preparative HPLC- General method A) gave the product as a white solid. LC (Analytical HPLC- General method A): 1.505 min. HRMS (ESI): calcd for $C_{15}H_{13}N_2O_3$ $[M+H]^+$ *m/z* 269.0921, found 269.0931. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.16 - 8.20 (m, 1H), 8.05 (d, *J* = 8.61 Hz, 2H), 7.94 (dd, *J* = 1.57, 8.61 Hz, 1H), 7.74 (d, *J* = 8.61 Hz, 1H), 7.01 (d, *J* = 8.61 Hz, 2H), 3.89 (s, 3H). Purity > 99%.

2,2-Bis((tosyloxy)methyl)propane-1,3-diyl bis(4-methylbenzenesulfonate) (39b). To a solution of 2,2-bis(hydroxymethyl)propane-1,3-diol (0.500 g, 3.67 mmol) in pyridine (8.9 mL) was added p-toluenesulfonyl chloride (3.14 g, 16.45 mmol) and the resulting mixture was stirred at room temperature for 48 h. Cold water was then added and the precipitate was collected by filtration, washed with cold water and dried in vacuo to give the title compound as a white solid (1.781 g, 2.366 mmol, 64%). LC (Analytical HPLC- General method A): 2.270 min. HRMS (ESI): calcd for $C_{33}H_{37}O_{12}S_4$ $[M+H]^+$ *m/z* 753.1162, found 753.1076. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.64 (d, *J* = 8.61 Hz, 8H), 7.46 (d, *J* = 8.22 Hz, 8H), 3.80 (s, 8H), 2.43 (s, 12H).

Methyl 2-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (40). A mixture of methyl 2-(4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**50**) (0.146 g, 0.423 mmol), 1-methylpiperazine (0.141 mL, 1.270 mmol), cesium carbonate (0.414 g, 1.270 mmol) and sodium iodide (0.063 g, 0.423 mmol) in DMF (10 mL) was heated at 100°C for 20 h. The cooled mixture was diluted with chloroform and the insolubles were removed by filtration. The filtrate was then concentrated to dryness and the residue was purified by flash chromatography [eluting with 0-40% dichloromethane-methanol-NH_4OH (50:45:5)-dichloromethane] to give the title compound as a white solid (0.096 g, 0.235 mmol, 56%). LC (Analytical HPLC- General method A): 1.348 min. HRMS (ESI): calcd for $C_{23}H_{29}N_4O_3$ $[M+H]^+$ *m/z* 409.2234, found 409.2199. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.05 - 8.15 (m, 3H), 7.80 (d, *J* = 8.61 Hz, 1H), 7.49 - 7.69 (m, 2H), 7.11 (d, *J* = 9.00 Hz, 2H), 4.08 (t, *J* = 6.26 Hz, 2H), 3.85 (s, 3H), 2.18 - 2.44 (m, 10H), 2.13 (s, 3H), 1.87 (quint, *J* = 6.85 Hz, 2H). Purity = 97%.

N-Benzyl-2-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxamide (41). 2-(4-(3-(4-Methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**51**) (0.025 g, 0.063 mmol) and benzylamine (0.015 mL, 0.133 mmol) were reacted according to General procedure D. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.011 g, 0.023 mmol, 36%). LC (Analytical HPLC- General method A): 1.513 min. HRMS (ESI): calcd for $C_{29}H_{34}N_5O_2$ $[M+H]^+$ *m/z* 484.2707, found 484.2717. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.11 (t, *J* = 6.06 Hz, 1H), 8.11 - 8.20 (m, 3H), 7.83 - 7.91 (m, 1H), 7.68 (d, *J* = 8.61 Hz, 1H), 7.30 - 7.38 (m, 4H), 7.21 - 7.29 (m, 1H), 7.18 (d, *J* = 9.00 Hz, 2H), 4.53 (d, *J* = 5.87 Hz, 2H), 4.16 (t, *J* = 6.26 Hz, 2H), 3.22 - 3.49 (m, 4H), 2.83 - 3.11 (m, 6H), 2.78 (br s, 3H), 1.95 - 2.11 (m, 2H). Purity = 90%.

2-(4-(3-(4-Methylpiperazin-1-yl)propoxy)phenyl)-N-(2-morpholinoethyl)-1H-benzo[d]imidazole-6-carboxamide (42). 2-(4-(3-(4-Methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**51**) (0.025 g, 0.063 mmol) and 2-morpholinoethanamine (0.017 mL, 0.133 mmol) were reacted according to General Method A. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.009 g, 0.018 mmol, 28%). LC (Analytical HPLC- General method A): 1.013 min. HRMS (ESI): calcd for $C_{28}H_{39}N_6O_3$ $[M+H]^+$ *m/z* 507.3078, found 507.3070. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.66 - 9.86 (m, 1H), 8.75 (t, *J* = 5.67 Hz, 1H), 8.07 - 8.18 (m, 3H), 7.77 (d, *J* = 9.39 Hz, 1H), 7.64 (d, *J* = 8.22 Hz, 1H), 7.14 (d, *J* = 9.00 Hz, 2H), 4.13 (t, *J* = 6.26 Hz, 2H), 3.84 - 4.06 (m, 4H), 3.54 - 3.84 (m, 12H), 3.26 - 3.40 (m, 2H), 3.13 (dt, *J* = 4.11, 7.53 Hz, 2H), 2.76 (br s, 3H), 1.93 - 2.06 (m, 2H), 1.14 - 1.28 (m, 2H). Purity = 95%.

2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxylic acid (43). A sealable tube was charged with 3-bromo-4-methoxybenzaldehyde (1.00 g, 4.65 mmol), 3,4-diaminobenzoic acid (0.708 g, 4.65 mmol), anhydrous DMF (20 mL) and sodium metabisulfite (0.884 g, 4.65 mmol), and the resulting mixture was heated at 80°C for 3 h. The cooled mixture was then concentrated to dryness and the crude residue obtained was partitioned with ethyl acetate-water. The organic layer was separated, dried over $MgSO_4$, filtered and concentrated to afford the crude title compound as a grey solid that was used as such in the next step (1.80 g, 5.18 mmol, 111%). LC (Analytical HPLC- General method A): 1.657 min. HRMS (ESI): calcd for $C_{15}H_{12}BrN_2O_3$ $[M+H]^+$ *m/z* 347.0026, found 347.0048. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.41 (d, *J* = 2.35 Hz, 1H),

8.20 (dd, *J* = 2.15, 8.80 Hz, 1H), 8.15 (s, 1H), 7.84 (dd, *J* = 1.76, 8.41 Hz, 1H), 7.64 (d, *J* = 8.22 Hz, 1H), 7.34 (d, *J* = 9.00 Hz, 1H), 3.95 (s, 3H). Purity > 99%.

2-(3-Bromo-4-methoxyphenyl)-*N*,*N*-diethyl-1H-benzo[d]imidazole-6-carboxamide (**44**). 2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**43**) (0.050 g, 0.144 mmol) and diethylamine hydrochloride (0.017 g, 0.158 mmol) were reacted according to General procedure E. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.034 g, 0.085 mmol, 59%). LC (Analytical HPLC- General method A): 1.727 min. HRMS (ESI): calcd for $C_{19}H_{21}BrN_3O_2$ $[M+H]^+$ *m/z* 402.0812, found 402.0830. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.42 (d, *J* = 1.96 Hz, 1H), 8.20 (dd, *J* = 2.35, 8.61 Hz, 1H), 7.67 (d, *J* = 8.22 Hz, 1H), 7.59 (s, 1H), 7.37 (d, *J* = 8.61 Hz, 1H), 7.26 (dd, *J* = 1.76, 8.41 Hz, 1H), 3.97 (s, 3H), 3.37 (br s, 4H), 1.13 (br s, 6H). Purity = 93%.

N-Benzyl-2-(3-bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxamide (**45**)**.** 2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**43**) (0.050 g, 0.144 mmol) and phenylethanamine (0.017 g, 0.158 mmol) were reacted according to General procedure E. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.028 g, 0.064 mmol, 45%). LC (Analytical HPLC- General method A): 1.867 min. HRMS (ESI): calcd for $C_{22}H_{19}BrN_3O_2$ $[M+H]^+$ *m/z* 436.0655, found 436.0655. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.12 (t, *J* = 6.06 Hz, 1H), 8.43 (d, *J* = 2.35 Hz, 1H), 8.21 (dd, *J* = 2.35, 8.61 Hz, 1H), 8.18 (s, 1H), 7.87 (dd, *J* = 1.76, 8.41 Hz, 1H), 7.69 (d, *J* = 8.22 Hz, 1H), 7.29 - 7.41 (m, 5H), 7.19 - 7.29 (m, 1H), 4.53 (d, *J* = 6.26 Hz, 2H), 3.97 (s, 3H). Purity = 98%.

(2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazol-6-yl)(4-methylpiperazin-1-yl)methanone (**46**)**.** 2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**43**) (0.050 g, 0.144 mmol) and 1-methylpiperazine (0.016 g, 0.158 mmol) were reacted according to General procedure E. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.028 g, 0.065 mmol, 45%). LC (Analytical HPLC- General method A): 1.301 min. HRMS (ESI): calcd for $C_{20}H_{22}BrN_4O_2$ $[M+H]^+$ *m/z* 429.0921, found 429.0946. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.99 (br s, 1H), 8.41 (d, *J* = 2.35 Hz, 1H), 8.19 (dd, *J* = 2.35, 8.61 Hz, 1H), 7.71 (s, 1H), 7.66 (d, *J* = 8.22 Hz, 1H), 7.28 - 7.36 (m, 2H), 3.94 (s, 3H), 3.21 - 3.50 (m, 6H), 3.02 - 3.14 (m, 2H), 2.82 (s, 3H). Purity > 99%.

2-(3-Bromo-4-methoxyphenyl)-N-(2-morpholinoethyl)-1H-benzo[d]imidazole-6-carboxamide (**47**)**.** 2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**43**) (0.050 g, 0.144 mmol) and 2-morpholinoethanamine (0.021 g, 0.158 mmol) were reacted according to General procedure E. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.030 g, 0.065 mmol, 45%). LC (Analytical HPLC- General method A): 1.415 min. HRMS (ESI): calcd for $C_{21}H_{24}BrN_4O_3$ $[M+H]^+$ *m/z* 459.1026, found 459.1017. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.77 (br s, 1H), 8.77 (t, *J* = 5.87 Hz, 1H), 8.42 (d, *J* = 2.35 Hz, 1H), 8.21 (dd, *J* = 2.35, 8.61 Hz, 1H), 8.14 (s, 1H), 7.79 (dd, *J* = 1.76, 8.41 Hz, 1H), 7.67 (d, *J* = 8.61 Hz, 1H), 7.35 (d, *J* = 8.61 Hz, 1H), 3.97 - 4.08 (m, 2H), 3.96 (s, 3H), 3.53 - 3.74 (m, 6H), 3.36 (t, *J* = 6.26 Hz, 2H), 3.17 (br s, 2H). Purity > 99%.

(2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazol-6-yl)(morpholino)methanone (**48**)**.** 2-(3-Bromo-4-methoxyphenyl)-1H-benzo[d]imidazole-6-carboxylic acid (**43**) (0.050 g, 0.144 mmol) and morpholine (0.014 g, 0.158 mmol) were reacted according to General procedure E. The crude residue obtained was purified by prep HPLC (Preparative HPLC- General method A) to give the title compound as a white solid (0.038 g, 0.091 mmol, 63%). LC (Analytical HPLC- General method A): 1.536 min. HRMS (ESI): calcd for $C_{19}H_{19}BrN_3O_3$ $[M+H]^+$ *m/z* 418.0589, found 418.0586. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.44 (d, *J* = 2.35 Hz, 1H), 8.21 (dd, *J* = 2.35, 8.61 Hz, 1H), 7.68 - 7.75 (m, 2H), 7.33 - 7.42 (m, 2H), 3.97 (s, 3H), 3.41 - 3.71 (m, 8H). Purity > 99%.

Methyl 2-(3-bromo-4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**49**)**.** To a solution of methyl 2-(3-bromo-4-hydroxyphenyl)-1H-benzo[d]imidazole-6-carboxylate (**34**) (0.300 g, 0.864 mmol) and cesium carbonate (0.422 g, 1.296 mmol) stirred in acetone (5.7 mL) was added 1-bromo-3-chloropropane (0.094 mL, 0.951 mmol). The resulting mixture was then stirred at 70°C for 16 h. After cooling, the reaction mixture was diluted with 15 mL of chloroform and the solids were removed by filtration. The filtrate was concentrated to dryness and the residue obtained was purified by flash chromatography (24 g cartridge) eluting with a gradient of ethyl acetate in dichloromethane (from 0 to 25%) to give the title compound as a white solid (0.097g, 0.229mmol, 27% yield). LC (Analytical HPLC- General method A): 2.048 min. HRMS (ESI): calcd for $C_{18}H_{17}BrClN_2O_3$ $[M+H]^+$ *m/z* 423.0106, 425.0084, found 423.0129, 425.0106. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.42 (d, *J* = 2.35 Hz, 1H), 8.15 - 8.22 (m, 2H), 7.87 (dd, *J* = 1.76,

8.41 Hz, 1H), 7.68 (d, *J* = 8.61 Hz, 1H), 7.39 (d, *J* = 8.61 Hz, 1H), 4.30 (t, *J* = 5.87 Hz, 2H), 3.83 - 3.90 (m, 5H), 2.25 (quint, *J* = 6.16 Hz, 2H).

Methyl 2-(4-(3-chloropropoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (50). To a solution of methyl 2-(4-hydroxyphenyl)-1H-benzo[d]imidazole-6-carboxylate (Scheme S6, Step a, **39a**; 0.800 g, 2.98 mmol) and cesium carbonate (1.457 g, 4.47 mmol) stirred in acetone (15 mL) was added 1-bromo-3-chloropropane (0.324 mL, 3.28 mmol) and the resulting mixture was heated at 80°C for 16 h. The cooled mixture was then concentrated to dryness and the residue was purified by flash chromatography (40 g cartridge, 0-5% MeOH-dichloromethane) to give the title compound as a white solid (0.340 g, 0.986 mmol, 33%). LC (Analytical HPLC- General method A): 1.824 min. HRMS (ESI): calcd for $C_{18}H_{18}ClN_2O_3$ $[M+H]^+$ *m/z* 345.1000, found 345.1021. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.08 - 8.16 (m, 3H), 7.83 (d, *J* = 7.43 Hz, 1H), 7.53 - 7.71 (m, 1H), 7.15 (d, *J* = 8.61 Hz, 2H), 4.19 (t, *J* = 6.06 Hz, 2H), 3.87 (s, 3H), 3.82 (t, *J* = 6.46 Hz, 2H), 2.21 (quint, *J* = 6.16 Hz, 2H).

2-(4-(3-(4-Methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylic acid (51). A solution of methyl 2-(4-(3-(4-methylpiperazin-1-yl)propoxy)phenyl)-1H-benzo[d]imidazole-6-carboxylate (**40**) (0.196 g, 0.480 mmol) and 1 M sodium hydroxide (1.44 mL, 1.44 mmol) in a mixture of methanol (1.44 mL) and THF (4.32 mL) was stirred with heating at 70°C for 1 h. The cooled mixture was concentrated to dryness and the crude residue obtained was treated with 1 M HCl (1.44 mL, 1.44 mmol). The resulting slurry was filtered, and the filter-cake was washed with water and then dried in vacuo to give the title compound as a solid. It was used as such for the next step without further purification. LC (Analytical HPLC- General method B): 0.992 min. MS (APCI): calcd for $C_{22}H_{26}N_4O_3$ $[M+H]^+$ *m/z* 395.2, found 395.2.

Table S1. Crystallographic data for DfrB1•**3** (PDB ID 6NXZ) and DfrB1•**1** (PDB ID 6NY0).

	DfrB1•**3** (PDB ID 6NXZ)	DfrB1•**1** (PDB ID 6NY0)
Data collection statistics		
Space group	$I4_122$	$I4_122$
Number of Molecules per Asymmetric Unit	1	1
a=b (Å)	67.617	67.492
c (Å)	51.977	51.791
$\alpha = \beta = \gamma$ (º)	90	90
Wavelength (Å)	1.54	0.98
Resolution Range (Å)[a]	50-1.75 (1.78-1.75)	50-1.40 (1.43-1.40)
Completeness (%)[a]	94.3 (48.7)	100.0 (100.0)
Redundancy [a]	79.2 (1.5)	20.1 (18.5)
R_{merge} (%)[a]	20.4 (>100)	7.6 (>100)
R_{meas} (%)[a]	20.5 (>100)	ND[b]
R_{pim} (%)[a]	2.0 (>100)	ND[b]
$CC_{1/2}$ [a]	(0.697)	ND[b]
CC* [a]	(0.907)	ND[b]
Refinement statistics		
Total number of reflections (reflections in R-free set)	5681 (319)	11,543 (624)
R_{factor} (%)	17.85	14.203
R_{free} (5% free test set) (%)	20.82	15.774
Number of atoms	515	562
Protein	429	459
Water	60	72
Buffer components	18	23
Inhibitor	8	8
RMSD		
Bond length (Å)	0.020	0.018
Bond angle (º)	2.061	2.076
Average atomic B-Factor (Å^2)	21.063	15.229
Protein (Å^2)	19.200	14.035
Water (Å^2)	33.637	26.110
Buffer components (Å^2)	70.420	37.496
Inhibitor (Å^2)	29.145	11.916
Ramachandran Plot	53	55
Residues in Favoured Positions	53	55
Residues in Allowed Positions	0	0
Residues in Disallowed Positions	0	0

[a] Items in parentheses refer to the highest resolution shell.

[b] Not determined; the version of HKL-2000 used to process these data did not report these statistics.

Table S2. Relative binding frequency per residue of DfrB1 with all inhibitors, resulting from the compilation of simulated poses (**3**, Figure S1). Inhibitors are listed in the order of their appearance in the Results section. Binding events were determined using the Protein Ligand Interaction Fingerprints (PLIF) module in MOE (CCG). The distribution of interaction frequency is gray-scaled from the highest frequency (■) to no binding observed (□).

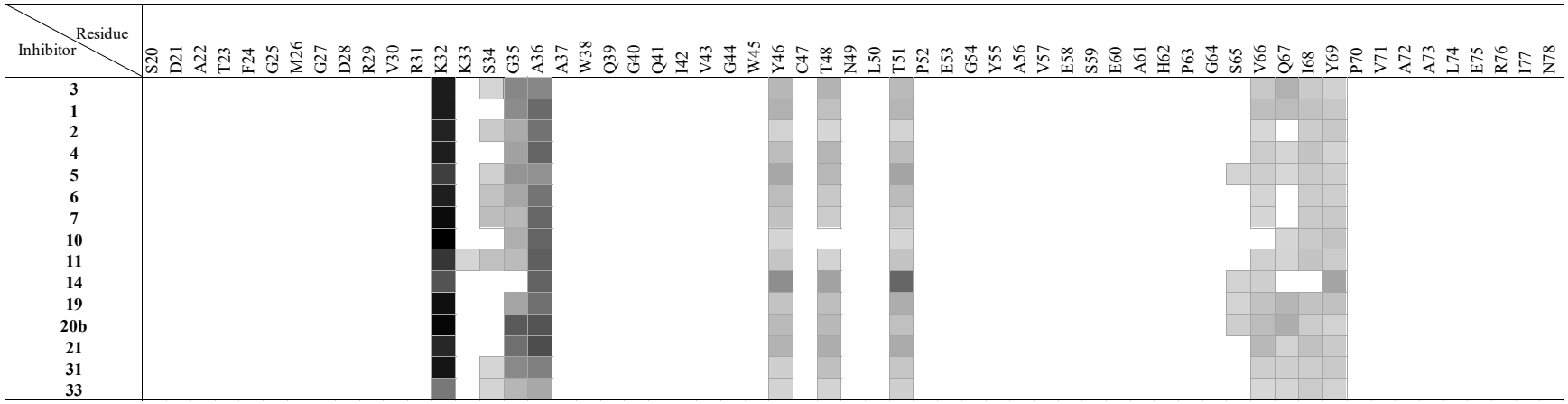


Table S3. Types of interactions between inhibitor segments and amino acids of DfrB1 determined using the Protein Ligand Interaction Fingerprints (PLIF) module in MOE (CCG). Inhibitors are listed in the order of their appearance in the Results section. H-bonds: D: side-chain donor, A: side-chain acceptor, d: backbone donor, a: backbone acceptor. I: ionic interaction. R: arene interaction.

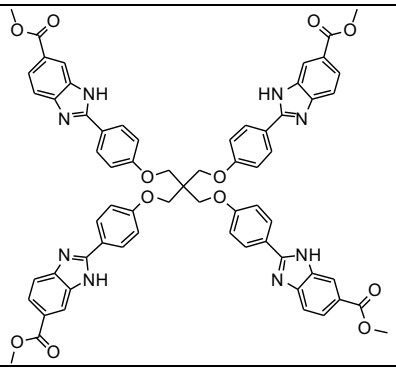
	Moiety	[...]	K32	K33	S34	G35	A36	[...]	Y46	C47	T48	N49	L50	T51	[...]	S65	V66	Q67	I68	Y69	[...]
3	Terminal carboxylate		DI		dD	d	d		D		D			D							
	Benzimidazole																Rd	A	a	R	
	Phenoxy																	D	R		
	Central core																a	AD	ad		
1	Terminal carboxylate		DI			d	d		D		D			D							
	Benzimidazole																ad		d	R	
	Phenoxy																	D	dR	R	
	Central core																a	A	ad		
2	Terminal carboxylate		DI		dD	d	d		D		D			D							
	Benzimidazole																aR		a	R	
	Phenoxy																d		R		
	Central core																				
4	Terminal carboxylate		DI			d	d		D		D			D							
	Benzimidazole																dR		a	R	
	Phenoxy																	D	R		
	Central core																				
5	Terminal carboxylate		DI		dD	d	d		D		D			D			d				
	Benzimidazole															R	ad		a	R	
	Phenoxy																d	DR	Rd		
	Central core																				
6	Terminal carboxylate		DI		D	d	d		D		D			D							
	Benzimidazole															R	adR		a	R	
	Phenoxy																	D	R		
	Central core																				
7	Terminal carboxylate		DI		D	d	d		D		D			D							
	Benzimidazole																d		Ra	R	
	Phenoxy																		R		
	Central core																				
10	Terminal carboxylate		DI			d	d		D					D							
	Benzimidazole																	D	aR	R	
	Phenoxy																	D	R		
	Central core																				
11	Terminal carboxylate		DI	I	dD	d	d		D		D			D							
	Benzimidazole																	AR	d	R	
	Phenoxy																d	DR	R		
	Central core																R		R		
14	Terminal carboxylate		DI				d		D		D			D							
	Benzimidazole																				
	Phenoxy															R	R			R	
	Central core																				
19	Terminal carboxylate		DI			d	d		D		D			D							
	Benzimidazole															R	R		ad	R	
	Phenoxy																		d		
	Central core																a	AD	d		
20b	Terminal carboxylate		DI			d	d		D		D			D							
	Benzimidazole																dRa			R	
	Phenoxy															A	a		Ra		
	Central core																a	AD	D		
21	Terminal carboxylate		DI			d	d		D		D			D							
	Benzimidazole																aRd	D	a	R	
	Phenoxy																a	A	R		
	Central core																				
31	Terminal carboxylate		DI		D	d	d		D		D			D							
	Benzimidazole																aR		a	R	
	Phenoxy																	DR	R	R	
	Central core																	A			
33	Terminal carboxylate		DI		Dd	d	d		D		D			D							
	Benzimidazole																ad	A	aR	R	
	Phenoxy																	D	R		
	Central core																	R		R	

Table S4. IC_{50} upon substitution of the terminal carboxylates.

Compound	IC_{50} (μM) [a]
22	> 50
23	> 50
24	> 50
25	> 30
26	> 100
27	> 100
28	> 100
29	> 50
30	> 100

[a] Compounds were tested at the highest possible concentration, considering constraints due to precipitation.

Table S5. The methyl ester of **31** does not inhibit DfrB1.

Compound	Structure	IC_{50} (μM)[a]
32		>50

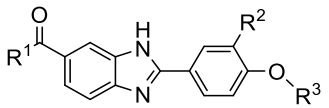
[a] Compound **32** was tested at the highest possible concentration, considering constraints due to precipitation.

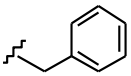
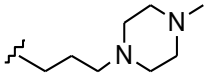
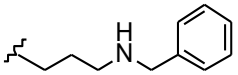
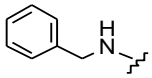
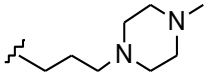
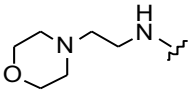
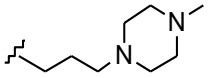
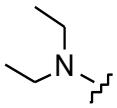
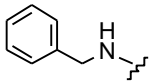
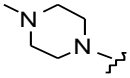
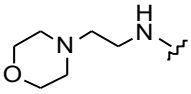
Table S6. Inhibition with representative non-symmetrical benzimidazole-based compounds. Table S8 lists nine further compounds in this category.

Compound	IC_{50} (μM) [a]
37	> 630
40	> 500
43	> 630
48	> 630

[a] Compounds were tested at the highest possible concentration, considering constraints due to precipitation.

Table S7. Inhibition with nine further non-symmetrical benzimidazole-based compounds.



#	R^1	R^2	R^3	IC_{50} (μM) [a]
34	-OMe	-Br	-H	> 30
35	-OMe	-Br		> 30
36	-OMe	-Br		> 630
38	-OMe	-Br		> 30
39a	-OMe	-H	-H	> 30
41		-H		> 500
42		-H		> 500
44		-Br	-Me	> 160
45		-Br	-Me	> 30
46		-Br	-Me	> 2,500
47		-Br	-Me	> 630

[a] Compounds were tested at the highest possible concentration, considering constraints due to precipitation.

Table S8. IC_{50} of selected inhibitors for DfrB1 and DfrB4.[a]

		IC_{50} (μM)				
	TMP	**1**	**3**	**33**	**20b**	**31**
DfrB1	$(19 \pm 4) \times 10^3$	130 ± 11[b]	113 ± 13	240 ± 87	83 ± 22	35 ± 9.0
DfrB4	$(12 \pm 2) \times 10^3$	69 ± 22	76 ± 18	210 ± 25	73 ± 13	37 ± 9.6

[a] Values are given as the average ± standard deviation from the mean of at least duplicates of triplicates.

[b] Value taken from Table 2 in [3].

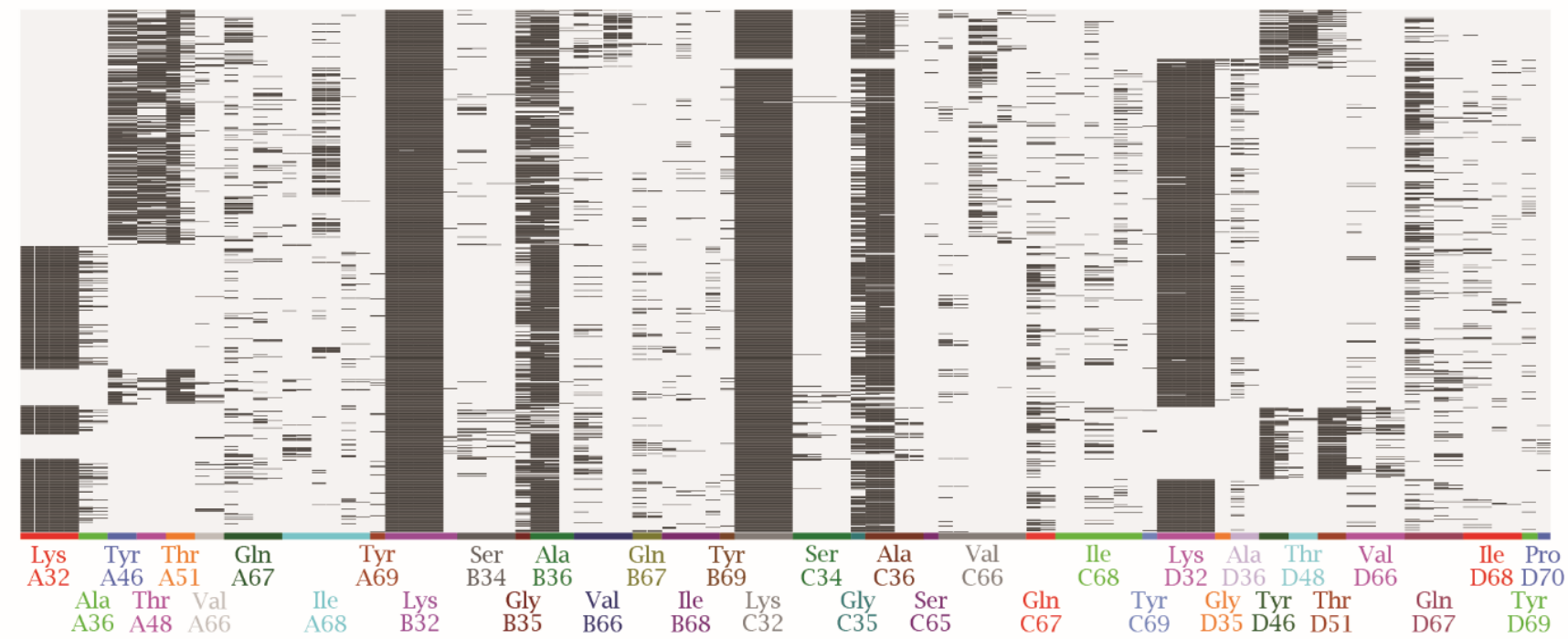


Figure S1. Matrix of the binding residues of DfrB1 with **3**. Each row represents one pose and each column, a specific interaction with a residue. For example, each of four Lys32 in the tetramer (Lys32A, Lys32B, Lys32C and Lys32D) can form 4 different interactions with the terminal carboxylate: side-chain H-bond1, side-chain H-bond2, side-chain ionic interaction1 and side-chain ionic interaction2. The letters A to D indicate the protomer implicated in binding. Each interaction is scored as a black mark.

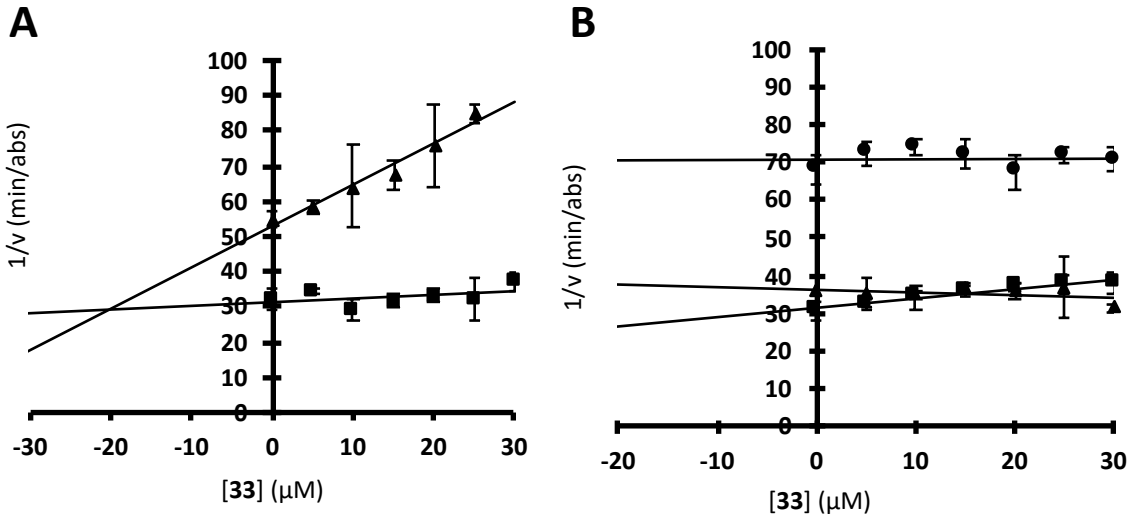


Figure S2. Dixon plots of **33** with DfrB1. The reciprocal velocity of DfrB1 as a function of inhibitor concentration determines the type of inhibition. The reciprocal velocity of DfrB1 as a function of inhibitor concentration determines the type of inhibition. (A) DHF was held constant and saturating at 50 μM and NADPH was 5 μM (~1 × K_M^{NADPH}; ▲) or 80 μM (22 × K_M^{NADPH}; ■). The intercept of the slopes gave K_i. (B) NADPH was held constant and saturating at 50 μM and DHF was 5μM (~2 × K_M^{DHF}; ●) 25 μM (13 × K_M^{DHF}; ▲) or 164 μM (86 × K_M^{DHF}; ■). Values are given as the mean ± standard deviation for triplicate results.

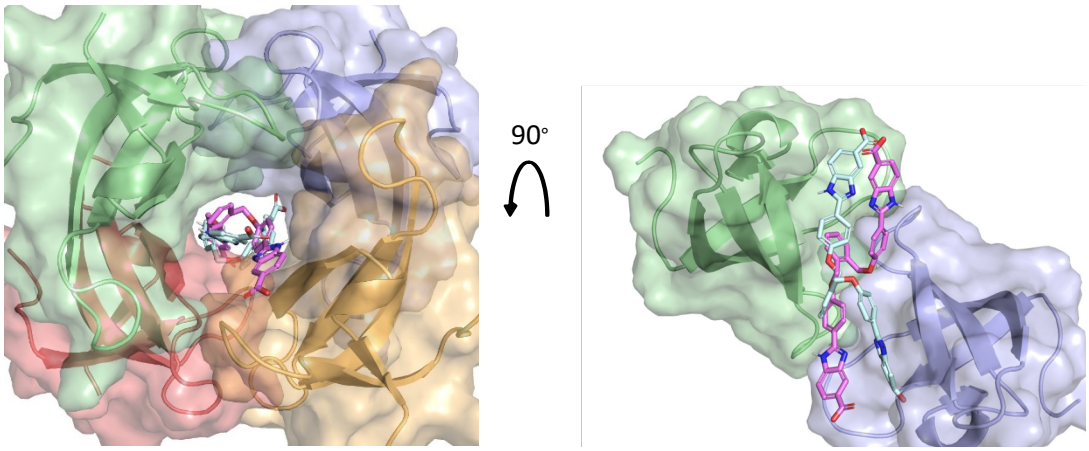


Figure S3. Theoretical representation of two molecules of **33** in the active site of DfrB1 (left) and from a top view (right). Contrary to other representations bound inhibitors, here only one molecule of **33** was modeled using LowMode MD simulations, consistent with the Hill coefficient of 1.1 (Figure 12). The bound **33** was then duplicated in a symmetry-related orientation to provide a

theoretical overlay of two molecules of **33** in the active site. Severe clashes are observed; the *o*-substituted central core of **33** adopts a wide twist that precludes fitting two molecules of **33** alongside each other in the tunnel.

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