Identification of an anti–MRSA dihydrofolate reductase inhibitor from a diversity–oriented synthesis

Emma E. Wyatt, Warren R. J. D. Galloway, Gemma L. Thomas, Martin Welch, Olivier Loiseleur, Alleyn T. Plowright, and David R. Spring.*

Supplementary Experimental Section

1.1 General experimental details

Reactions were performed using oven-dried glassware apparatus under an atmosphere of nitrogen. Anhydrous dimethylformamide (DMF) and ethanol (EtOH), when used as obtained from commercial sources. All other reagents were used as obtained from commercial sources. All reactions were monitored by thin layer chromatography (TLC) performed on commercially prepared glass plates precoated with Merck silica gel 60 F254 or aluminium oxide 60 F254. Visualisation was by the quenching of UV fluorescence (ν_{max} = 254 nm) or by staining with ceric ammonium molybdate, potassium permanganate or Dragendorff 's reagent (0.08% w/v bismuth subnitrate and 2% w/v KI in 3M aq. AcOH). Retention factors (R_f) are quoted to 0.01. Yields refer to spectroscopically pure compounds.

Infrared spectra were recorded neat on a Perkin-Elmer Spectrum One spectrometer with internal referencing. Selected absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}) and the following abbreviations are used: w, weak; m, medium; st, strong; br, broad.

Melting points were obtained using a Reichert hot plate microscope with a digital thermometer attachment and are uncorrected.

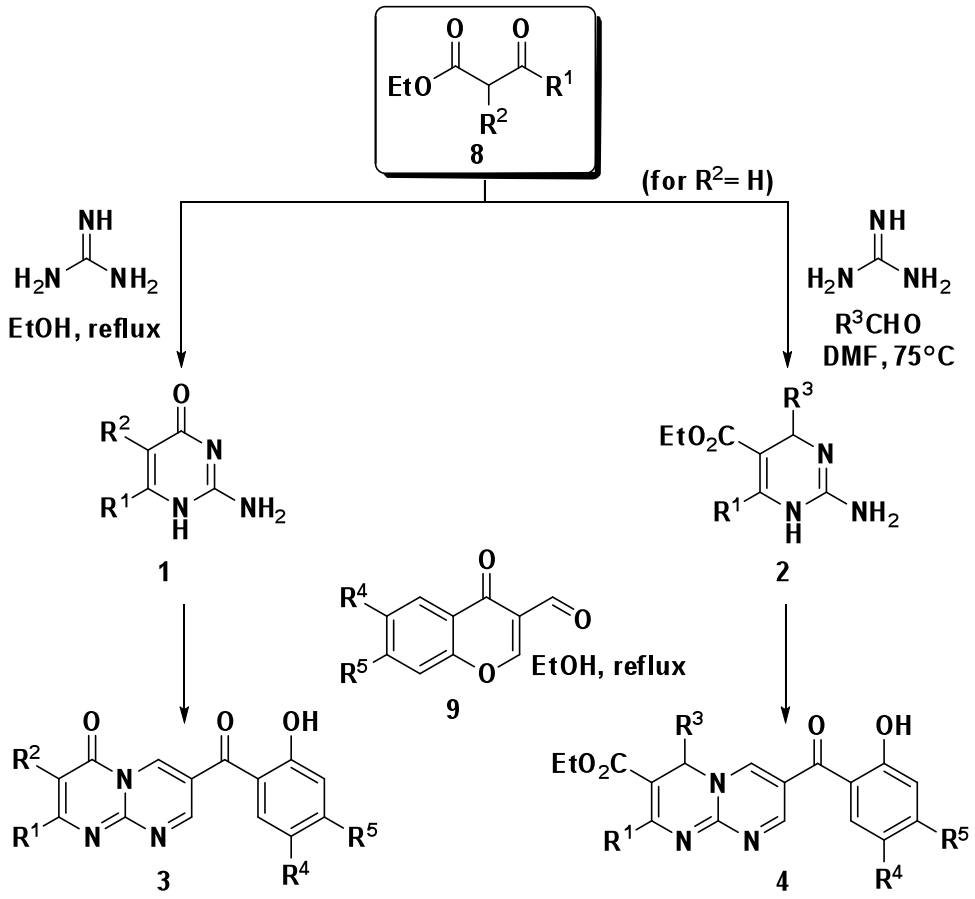
Proton magnetic resonance spectra were recorded using an internal deuterium lock at ambient probe temperatures (unless otherwise stated) on the following instruments: Bruker DPX-400 (400 MHz), Bruker Avance 400 QNP (400 MHz), Bruker Avance 500 BB ATM (500 MHz) and Bruker Avance 500 Cryo Ultrashield (500 MHz). Chemical shifts (δ_H) are quoted in ppm, to the nearest 0.01 ppm, and are referenced to the residual non-deuterated solvent peak. Coupling constants (*J*) are reported in Hertz to the nearest 0.5 Hz. Data are reported as follows: chemical shift, integration, multiplicity [br, broad; s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; sept, septet; m, multiplet; or as a combination of these (*e.g.* dd, dt, *etc.*)], coupling constant(s) and assignment. Proton assignments were determined either on the basis of unambiguous chemical shift or coupling pattern, by patterns observed in 2D experiments (^1H-^1H COSY, HMBC and HMQC) or by analogy to fully interpreted spectra for related compounds.

Carbon magnetic resonance spectra were recorded by broadband proton spin decoupling at ambient probe temperatures (unless otherwise stated) using an internal deuterium lock on the following instruments: Bruker DPX-400 (100 MHz), Bruker Avance 400 QNP (100 MHz), Bruker Avance 500 BB ATM (125 MHz) and Bruker Avance 500 Cryo Ultrashield (125 MHz). Chemical shifts (δ_C) are quoted in ppm, to the nearest 0.1 ppm, and are referenced to the residual non-deuterated solvent peak. Where appropriate, coupling constants are reported in Hertz to the nearest 0.5 Hz and data are reported as for proton magnetic resonance spectra without integration. Assignments were supported by DEPT editing and determined either on the basis of unambiguous chemical shift or coupling pattern, by patterns observed in 2D experiments (HMBC and HMQC) or by analogy to fully interpreted spectra for related compounds.

High resolution mass spectroscopy measurements were made by the EPSRC mass spectrometry service (Swansea) or recorded in-house using a Waters LCT Premier Mass Spectrometer or a Micromass Quadrapole-Time of Flight (Q-ToF) spectrometer. Mass values are reported within the error limits of ±5 ppm mass units. ESI = electrospray ionisation.

1.2 General experimental procedures for analogue synthesis

1.2.1 Outline of synthetic pathways used for analogue synthesis



Scheme 1: Outline of the synthetic routes used in analogue synthesis.

A variety of different β-keto esters **(8)** could serve as key building blocks for two main branching reactions. Reaction with guanidine would generate the corresponding amino pyrimidinones **1**[1] whereas reaction with a variety of aldehydes in the presence of guanidine would furnish dihydropyrimidine derivatives **2** *via* a three-component Biginelli-type reaction.[2] These structures could then be further modified by reaction with a range of substituted 3-formyl chromones[3] **(9)** to form bicylic nitrogen heterocyclic systems **3** and **4** (Scheme 1).

1.2.2 Building-blocks used in analogue synthesis

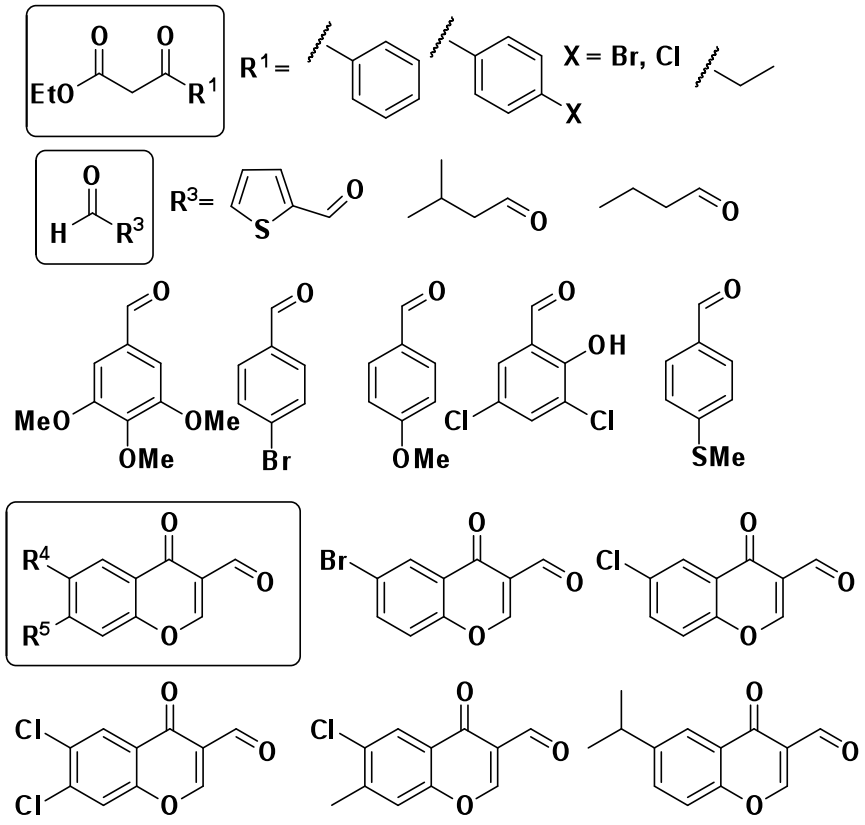


Figure 1: Building-blocks used for analogue synthesis.

Various combinations of substituted β−keto ester, aldehydes and chromone 'building-blocks' (Figure 1) were used in the general synthetic routes outlined in Scheme 1 to synthesize a small, focused collection of analogues based around heterocyclic frameworks **1-4** (See Appendix 1 for full list of compounds).

1.2.3 General method for the synthesis of pyrimidinones 1

A solution of β-ketoester (1 equiv.) and guanidine carbonate (1 equiv.) in EtOH was heated to reflux for 5 hrs. The resultant precipitate was collected by filtration, washed with cold EtOH, H_2O and acetone and dried under vacuum to give the desired product as a white solid.

1.2.4 General method for the synthesis of pyrimidine derivatives 2 *via* a three-component Biginelli reaction

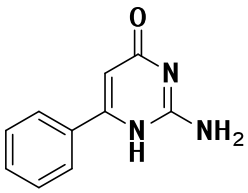
To a suspension of guanidine hydrochloride (1.2 equiv.) and sodium bicarbonate (4 equiv.) in anhydrous DMF was added the appropriate β-ketoester (1.1 equiv) and aldehyde (1 equiv.). The mixture was heated to 70 °C for 16 hrs. After cooling to room temperature the solution was poured onto crushed ice to facilitate precipitation and the yellow solid was collected by filtration.

1.2.5 General method for the reaction with chromones (formation of bicyclic species 3 and 4)

A suspension of dihydropyrimidine (1 equiv.) and the appropriate chromone (1 equiv.) in EtOH was heated to reflux for 5 hrs. After cooling to room temperature the yellow solid was collected by filtration, washed with EtOH and dried under vacuum to give the desired product

1.3 Representative compounds

2-Amino-6-phenyl-1*H*-pyrimidin-4-one (10)



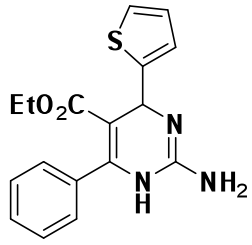
10

A solution of ethylbenzoyl acetate (100 mg, 0.520 mmol, 1 equiv.) and guanidine carbonate (94 mg, 0.520 mmol, 1 equiv.) in EtOH was heated to reflux for 5 hrs. The resultant precipitate was collected by filtration, washed with cold EtOH, H_2O and acetone and dried under vacuum to give the desired product as a white solid (60.6 mg, 0.324 mmol, 62 %).

ν_{max} (neat)/cm^{-1} 3336 m (N-H), 3060 m (N-H), 1643 m (amide), 1559 st (NH_2 bend), 1515 m (C=C); δ_H (500 MHz; DMSO) 10.85 (1H, br s, NH), 7.93 (2H, t, *J* 3.5 Hz, aryl CH), 7.43 (3H, m, aryl CH), 6.61 (2H, br s, NH_2), 6.10 (1H, s, CH); δ_C (100 MHz; DMSO) 163.3 (C), 162.5 (C), 155.7 (C), 137.2 (C), 129.8 (CH), 128.2 (CH), 126.5 (CH), 97.4 (CH); **HRMS** (ESI^+) *m/z* found 188.0819 $[M-H]^+$, $C_{10}H_{10}N_3O^+$ required 188.0819; **m.p.** 281-286 °C (EtOH) (lit.,[1] 312 °C).

This data is in agreement with that previously reported.[1]

Ethyl-2-amino-6-phenyl-4-thiophen-2-yl-1,4-dihydro-pyrimidine-5-carboxylate (11)



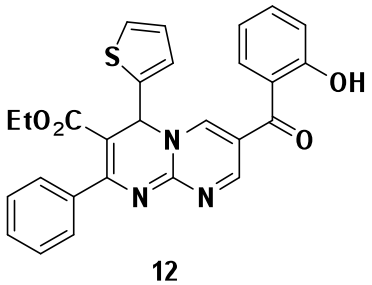
11

To a suspension of guanidine hydrochloride (542 mg, 5.68 mmol, 1.2 equiv.) and sodium bicarbonate (1.59 g, 18.9 mmol, 4 equiv.) in DMF was added ethylbenzoylacetate (0.91 cm^3, 5.20 mmol, 1.1 equiv) and 2-thiopenecarboxaldehyde (0.44 cm^3, 4.73 mmol, 1 equiv.). The mixture was heated to 70 °C for 16 hrs. After cooling to room temperature the solution was poured onto crushed ice to facilitate precipitation and the yellow solid was collected by filtration (1.31 g, 4.00 mmol, 85 %).

ν_{max} (neat)/cm^{-1} 3398 w (N-H), 3357 w (N-H), 1671 m (amide), 1579 m (NH_2 bend), 1529 w (C=C), 1230 st (C=O); δ_H (500 MHz; DMSO) 7.64 (1H, br s, NH), 7.35 (1H, dd, *J* 4.0, 1.0 Hz, aryl CH), 7.25 (5H, m, aryl CH), 7.00 (2H, m, aryl CH), 6.39 (1H, br s, NH_2), 5.58 (1H, s, CH), 3.74 (2H, q, *J* 7.0 Hz, CH_2CH_3), 0.80 (3H, t, *J* 7.07 Hz, CH_2CH_3); δ_C (125 MHz; DMSO) 165.6 (C), 155.2 (C), 150.6 (C), 128.0 (CH), 126.9 (CH), 126.6 (CH), 126.3 (CH), 124.3 (CH), 122.9 (CH), 97.8 (C), 58.0 (CH_2), 48.4 (CH), 13.6 (CH_3); **HRMS** (ESI^+) *m/z* found 328.1103 $[M-H]^+$, $C_{17}H_{18}N_3O_2S^+$ required 328.1120; **m.p.** 247-252 °C (DMF:H_2O) (lit.,[3] 223-225 °C).

This data is in agreement with that previously reported.[3]

Ethyl-7-(2-Hydroxy-benzoyl)-2-phenyl-4-thiophen-2-yl-4*H*-pyrimido[1,2-*a*]pyrimidine-3-carboxylate (12)



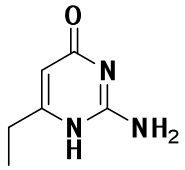
12

A suspension of **11** (1 g, 3.05 mmol, 1 equiv.) and 3-formylchromone (538 mg, 3.05 mmol, 1 equiv.) in EtOH was heated to reflux for 5 hrs. After cooling to room temperature the yellow solid was collected by filtration, washed with EtOH and dried under vacuum to yield the title compound product (745 mg, 1.54 mmol, 50 %).

ν_{max} (neat)/cm^{-1} 2753 br (O-H), 1694 m (ester), 1664 st (ketone), 1622 m (C=C), 1531 m (C=N); δ_H (500 MHz; DMSO) 11.84 (1H, br s, OH), 7.90 (1H, dd, *J* 6.5, 1.0 Hz, aryl CH), 7.51 (2H, m, aryl CH), 7.44 (2H, t, *J* 7.5 Hz, aryl CH), 7.38 (2H, m, aryl CH), 7.30 (1H, d, *J* 5.0 Hz, aryl CH), 7.0 (1H, d, *J* 3.0 Hz, aryl CH), 7.10 (1H, t, *J* 7.5 Hz, aryl CH), 7.06 (1H, d, *J* 8.0 Hz, aryl CH), 6.96 (1H, dd, *J* 5.0, 3.5 Hz, aryl CH), 6.23 (1H, s, N=CH), 6.17 (1H, s, N-CH=C), 5.45 (1H, s, CH), 3.90 (2H, qd, *J* 7.0, 3.0 Hz, CH_2CH_3), 0.80 (2H, t, *J* 7.0 Hz, CH_2CH_3); δ_C (125 MHz; DMSO) 179.5 (C), 164.6 (C), 155.9 (C), 148.6 (C), 140.1 (CH), 135.7 (CH), 135.1 (C), 130.2 (CH), 128.5 (CH), 128.2 (CH), 127.4 (CH), 127.0 (CH), 126.7 (CH), 126.6 (CH), 124.1 (C), 122.6 (CH), 118.1 (CH), 108.8 (C), 103.4 (C), 82.9 (CH), 60.3 (CH_2), 52.9 (CH), 13.5 (CH_3); **HRMS** (ESI$^+$) *m/z* found 484.1326 $[M-H]^+$, $C_{27}H_{22}N_3O_4S^+$ required 484.1331; **m.p.** 232-245 °C (EtOH) (lit.,[3] 254-257 C).

This data is consistent with that previously reported.[3]

2-Amino-6-ethylpyrimidin-4(1H)-one (13)

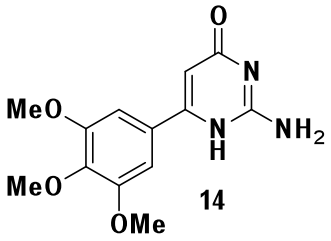


13

A suspension of ethylpropionylacetate (500 mg, 3.47 mmol, 1 equiv.) and guanidine carbonate (625 mg, 3.47 mmol, 1 equiv.) in EtOH (5 cm^3) was heated to reflux for 5 hrs. Gradual dissolution was followed by precipitation of a white solid, which was removed by filtration, washed with cold EtOH, H_2O and acetone and dried under vacuum to give the desired product as a white solid (163 mg, 1.17 mmol, 34 %).

ν_{max} (neat)/cm^{-1} 3320 m (N-H), 3180 m (N-H), 1646 st (amide), 1576 m (NH_2 bend), 1525 m (C=C); δ_H (500 MHz; MeOD) 5.59 (1H, s, C<u>H</u>), 4.90 (2H, s, N<u>H</u>$_2$), 2.37 (2H, q, *J* 7.5 Hz, C<u>H</u>$_2$CH$_3$), 1.18 (3H, q, *J* 7.5 Hz, CH$_2$C<u>H</u>$_3$); δ_C (125 MHz; MeOD) 174.1 (C), 170.7 (C), 161.7 (C), 100.2 (CH), 30.7 (CH_2), 13.1 (CH_3); **HRMS** (ESI^+) *m/z* found 162.0645 $[MNa]^+$, $C_6H_9N_3ONa^+$ required 162.0643; **m.p.** 250-254 °C (EtOH).

2-Amino-6-(3,4,5-trimethoxy-phenyl)-1*H*-pyrimidin-4-one (14)

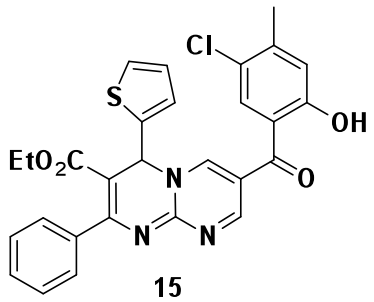


14

A suspension of ethyl-2-oxocyclohexane carboxylate (0.090 cm^3, 0.586 mmol, 2 equiv.) and guanidine carbonate (53.0 mg, 0.588 mmol, 1 equiv.) in EtOH (2 cm^3) was heated to reflux for 14 hrs. Gradual dissolution was followed by precipitation of a white solid, which was removed by filtration, washed with cold EtOH, H_2O and acetone and dried under vacuum to give the desired product as a white solid (45.2 mg, 0.274 mmol, 93 %).

ν_{max} (neat)/cm^{-1} 3390 m (N-H), 3111 m (N-H), 1673 m (amide), 1631 m (NH_2 bend), 1578 m (C=C); δ_H (500 MHz; DMSO) 10.78 (1H, br s, NH), 7.24 (2H, s, aryl H), 6.59 (2H, br s, NH_2), 6.18 (1H, m, C=CH), 3.83 (6H, s, 2 × CH_3), 3.70 (3H, s, CH_3); δ_C (125 MHz; DMSO) 175.8 (C), 158.6 (C), 152.6 (C), 103.7 (CH), 97.0 (CH), 60.0 (CH_3), 55.9 (CH_3); **HRMS** (ESI$^+$) *m/z* found 278.1132 $[M-H]^+$, $C_{13}H_{16}N_3O_4^+$ required 278.1135; **m.p.** 169-174 °C (EtOH).

3-Ethyl-7-(5-chloro-2-hydroxy-4-methyl-benzoyl)-2-phenyl-4-thiophen-2-yl-4*H*-pyrimido[1,2-*a*]pyrimidine-3-carboxylate (15)

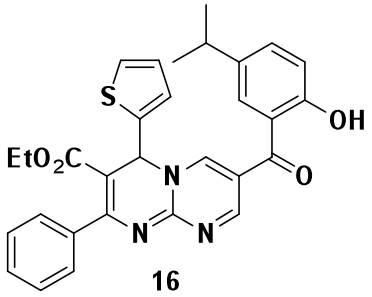


15

A suspension of **11** (25.0 mg, 0.0764 mmol, 1 equiv.) and 6-chloro-3-formyl-7-methylchromone (17.0 mg, 0764 mmol, 1 equiv.) in ethanol was heated to reflux for 6 hrs. Gradual dissolution was followed by rapid precipitation of a yellow solid. After cooling to 0 °C the precipitate was collected by filtration, washed with EtOH and dried under vacuum to give the desired product (34.2 mg, 0.0643 mmol, 84 %).

ν_{max} (neat)/cm^{-1} 2729 br (O-H), 1666 m (ester), 1624 m (ketone), 1541 m (C=N), 1504 w (C=C), 1234 st (C-O); δ_H (500 MHz; DMSO) 10.99 (1H, br s, OH), 7.68 (1H, s, aryl CH), 7.55 (2H, m, aryl CH), 7.40 (6H, m, aryl CH), 7.25 (1H, d, *J* 10.0 Hz, aryl CH), 7.02 (1H, s, N=CH), 6.50 (1H, s, N-CH=C), 6.03 (1H, s, CH), 3.83 (2H, m, CH_2CH_3), 2.37 (1H, s, CH_3), 0.78 (3H, t, *J* 7.0 Hz, CH_2CH_3); δ_C (125 MHz; DMSO) 177.7 (C), 164.6 (C), 154.5 (C), 144.2 (C), 142.5 (C), 129.1 (CH), 128.8 (CH), 128.8 (C), 128.6 (CH), 127.7 (CH), 127.3 (CH), 126.9 (C), 126.2 (C), 126.1 (C), 123.0 (CH), 121.0 (CH), 103.1 (C), 83.6 (CH), 83.2 (C), 59.8 (CH_2), 52.6 (CH), 20.2 (CH_3), 13.5 (CH_3); **HRMS** (ESI$^+$) *m/z* found 532.1087 $[M-H]^+$, $C_{28}H_{23}ClN_3O_4S^+$ required 532.1092; **m.p.** 273-277 °C (EtOH).

3-Ethyl-7-(2-hydroxy-5-isopropyl-benzoyl)-2-phenyl-4-thiophen-2-yl-4*H*-pyrimido[1,2-*a*]pyrimidine-3-carboxylate (16)

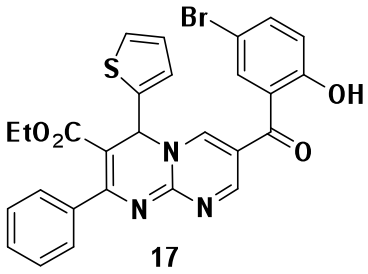


16

A suspension of **11** (25.0 mg, 0.0764 mmol, 1 equiv.) and 3-formyl-6-isopropyl chromone (17.0 mg, 0.0764 mmol, 1 equiv.) in EtOH was heated to reflux for 6 hrs. Gradual dissolution was followed by rapid precipitation of a yellow solid. After cooling to 0 °C the precipitate was collected by filtration, washed with EtOH and dried under vacuum to give the desired product (14.7 mg, 0.0280 mmol, 37 %).

ν_{max} (neat)/cm^{-1} 2720 br (O-H), 1675 m (ester), 1621 m (ketone), 1599 m (C=C), 1541 m (C=N), 1243 st (C-O); δ_H (500 MHz; DMSO) 10.88 (1H, br s, OH), 7.63 (1H, m, aryl CH), 7.53 (2H, m, aryl CH), 7.36 (6H, m, aryl CH), 7.24 (1H, d, *J* 3.0 Hz, aryl CH), 7.12 (1H, d, *J* 8.5 Hz, aryl CH), 7.01 (1H, t, *J* 4.0 Hz, aryl CH), 6.44 (1H, s, N=CH), 6.04 (1H, s, N-CH), 3.83 (2H, q, *J* 7.0 Hz, CH_2CH_3), 2.93 (1H, septuplet, *J* 7.0 Hz, CH_3CHCH_3), 1.20 (6H, d, *J* 7.0 Hz, CH_3CHCH_3), 0.78 (3H, t, *J* 7.0 Hz, CH_2CH_3); δ_C (125 MHz; $CDCl_3$) 179.0 (C), 164.6 (C), 154.3 (C), 142.6 (C), 134.8 (C), 134.5 (CH), 129.0 (CH), 128.5 (CH), 127.7 (CH), 127.3 (CH), 127.2 (CH), 126.8 (CH), 126.4 (C), 125.6 (C), 125.4 (C), 123.8 (CH), 123.4 (CH), 118.4 (CH), 108.2 (C), 103.1 (C), 82.8 (CH), 59.7 (CH_2), 59.3 (CH), 52.5 (CH), 32.8 (CH), 24.0 (CH_3), 23.9 (CH_3), 13.5 (CH_3); **HRMS** (ESI^+) *m/z* found 526.1799 $[M-H]^+$, $C_{30}H_{28}N_3O_4S^+$ required 526.1801; **m.p.** 247-252 °C (EtOH).

3-Ethyl-7-(5-bromo-2-hydroxy-benzoyl)-2-phenyl-4-thiophen-2-yl-4*H*-pyrimido[1,2-*a*]pyrimidine-3-carboxylate (17)

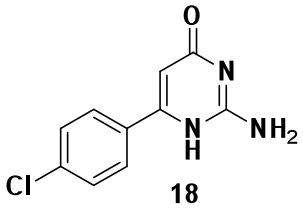


17

A suspension of **11** (25.0 mg, 0.0764 mmol, 1 equiv.) and 6-bromo-3-formyl chromone (19.0 mg, 0.0764 mmol, 1 equiv.) in EtOH was heated to reflux for 6 hrs. Gradual dissolution was followed by rapid precipitation of a yellow solid. After cooling to 0 °C the precipitate was collected by filtration, washed with EtOH and dried under vacuum to give the title compound (11.9 mg, 0.0212 mmol, 28 %).

ν_{max} (neat)/cm^{-1} 2751 br (O-H), 1671 m (ester), 1627 m (ketone), 1597 m (C=C), 1544 m (C=N), 1240 st (C-O); δ_H (500 MHz; DMSO) 11.03 (1H, br s, OH), 7.83 (1H, s, aryl CH), 7.77 (1H, d, *J* 8.0 Hz, aryl CH), 7.54 (1H, m, aryl CH), 7.41 (6H, m, aryl CH), 7.25 (1H, s, aryl CH), 7.17 (1H, m, aryl CH), 7.02 (1H, t, *J* 4.0 Hz, aryl CH), 6.55 (1H, s, N=CH), 6.05 (1H, s, N-CH), 3.83 (2H, m, CH_2CH_3), 0.78 (3H, t, *J* 7.0 Hz, CH_2CH_3); δ_C (125 MHz; DMSO) 177.8 (C), 164.5 (C), 155.2 (C), 148.5 (C), 142.5 (C), 138.2 (CH), 129.2 (CH), 129.1 (CH), 128.8 (CH), 128.6 (CH), 128.3 (CH), 128.1 (CH), 127.8 (CH), 127.3 (CH), 126.9 (CH), 126.2 (CH), 125.5 (C), 121.2 (CH), 114.2 (C), 103.4 (C), 83.8 (CH), 59.8 (CH_2), 52.7 (CH), 13.5 (CH_3); **HRMS** (ESI$^+$) *m/z* found 562.0433 $[M-H]^+$, $C_{27}H_{21}N_3O_4S^+$ required 562.0431; **m.p.** 259-264 °C (EtOH).

2-Amino-6-(4-chloro-phenyl)-1*H*-pyrimidin-4-one (18)



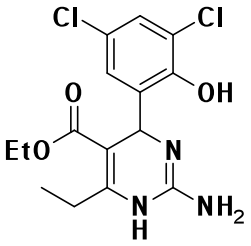
18

A solution of ethyl (4-chlorobenzoyl)acetate (100 mg, 0.441 mmol, 1 equiv.) and guanidine carbonate (79.5 mg, 0.441 mmol, 1 equiv.) in EtOH was heated to reflux for 3 hrs. The solution was cooled to 0 °C and the resultant precipitate was collected by filtration, washed with cold EtOH, H_2O and acetone and dried under vacuum to give the desired product as a white solid (97.7 mg, 0.441 mmol, 100 %).

ν_{max} (neat)/cm^{-1} 3332 m (N-H), 3050 m (N-H), 1655 st (amide), 1495 m (NH_2 bend), 1471 st (C=C); δ_H (500 MHz; DMSO) 10.84 (1H, br s, NH), 7.96 (2H, d, *J* 8.5 Hz, aryl CH), 7.49 (2H, d, *J* 8.5 Hz, aryl CH), 6.63 (2H, br s, NH_2), 6.13 (1H, s, CH); δ_C (100 MHz; DMSO) 163.7 (C), 161.7 (C), 156.2 (C), 136.6 (C), 135.1 (C), 128.89 (CH), 128.85 (CH), 98.2 (CH); **HRMS** (ESI^+) *m/z* found 222.0426 $[M-H]^+$, $C_{10}H_9ClN_3O^+$ required 222.0434; **m.p.** 309-311 °C (EtOH).

1.4 Synthesis of emmacin

(Ethyl-2-amino-4-(3,5-dichloro-2-hydroxy-phenyl)-6-ethyl-1,4-dihydro-pyrimidine-5-carboxylate)

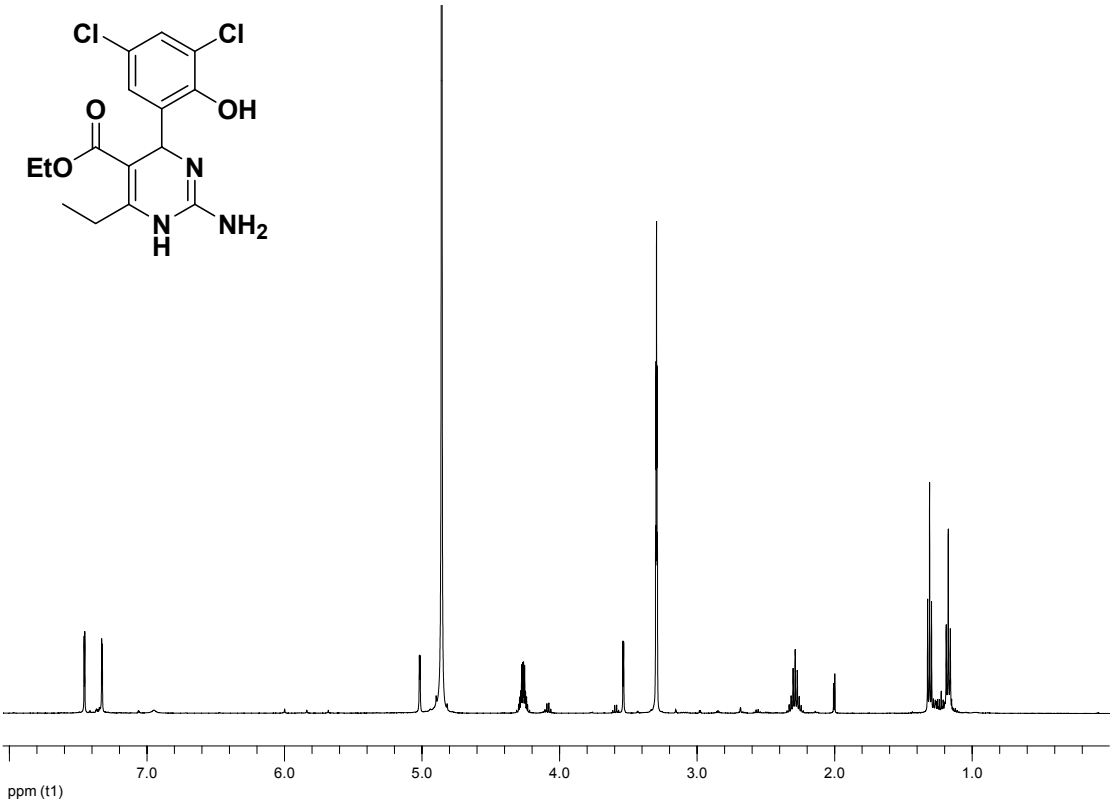


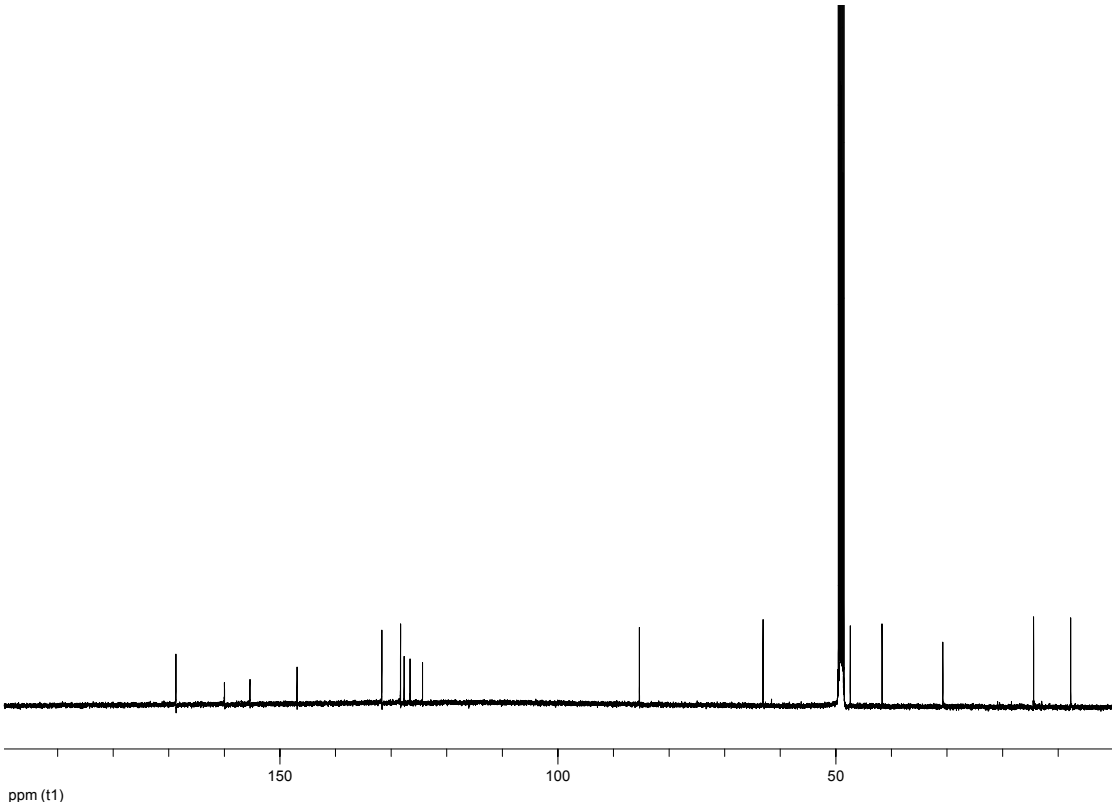
emmacin

To a suspension of guanidine carbonate (1 g, 7.57 mmol, 1.2 equiv.) and sodium bicarbonate (2.12 g, 25.2 mmol, 4 equiv.) in anhydrous DMF was added ethylpropionylacetate (0.99 cm^3, 6.94 mmol, 1.1 equiv.) and 3,5-dichlorosalicyl aldehyde (1.20 g, 6.31 mmol, 1.0 equiv.). The mixture was heated to 70 °C for 16 hrs. After cooling to room temperature the solution was poured onto crushed ice to facilitate precipitation and the yellow solid was collected by filtration (1.19 g, 3.32 mmol, 48 %).

ν_{max} (neat)/cm^{-1} 3291 br (N-H), 3151 br (O-H), 1732 m (ester), 1663 st (C=N), 1635 m (C=C), 1597 w (NH_2 bend), 1191 m (C-O); δ_H (500 MHz; MeOD) 7.45 (1H, d, *J* 2.5 Hz, aryl H), 7.33 (1H, d, *J* 2.5 Hz, aryl H), 5.02 (1H, d, CH), 3.74 (2H, m, $CO_2C\underline{H}_2CH_3$), 3.54 (1H, d, *J* 3.0 Hz, NH), 2.29 (2H, septet, *J* 7.5 Hz, $C\underline{H}_2CH_3$), 1.31 (3H, t, *J* 7.0 Hz, $CO_2CH_2C\underline{H}_3$), 1.17 (3H, t, *J* 7.5 Hz, $CH_2C\underline{H}_3$); δ_C (125 MHz; MeOD) 168.7 (C), 160.0 (C), 155.4 (C), 146.9 (C), 131.6 (CH), 128.3 (CH), 127.6 (C), 126.6 (C), 124.3 (C), 85.3 (C), 63.1 (CH_2), 41.7 (CH), 30.7 (CH_2) 14.4 (CH_3), 7.7 (CH_3); **HRMS** (ESI^+) *m/z* found 358.0710 $[M-H]^+$, $C_{15}H_{18}Cl_2N_3O_3^+$ required 358.0725; **m.p.** 215-219 °C (DMF:H_2O).

1.5 ^{1}H and ^{13}C NMR spectra of emmacin





1.6 Bacterial growth assays

1.6.1 Materials

- Compound – Library compounds dissolved in DMSO. Stock solutions were stored as 100 mM solutions.

- Luria Broth – 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0.

- EMRSA 15 and EMRSA 16 – Kindly supplied by Drs N. Brown and D. Brown, Addenbrookes Hospital HPA, Cambridge.

- Spectrophotometric Determination – Experiment progress was monitored in a BioRad plate reader. The plates were shaken for 3 seconds prior to optical density reading at 595 nm.

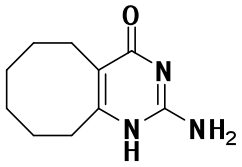
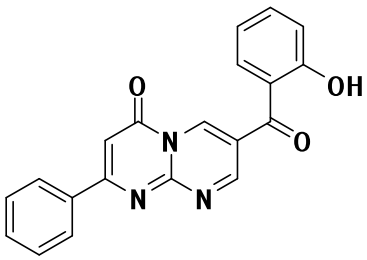
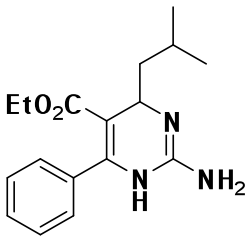
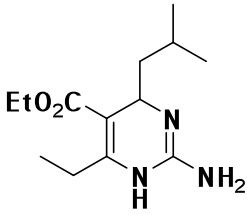
1.6.2 Method

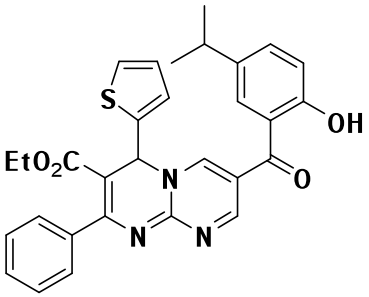
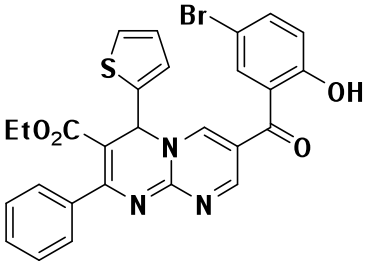
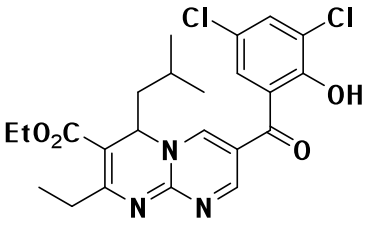
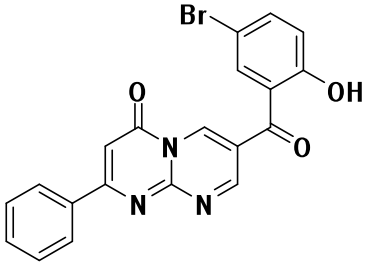
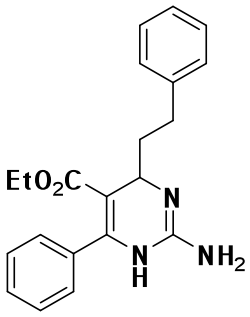
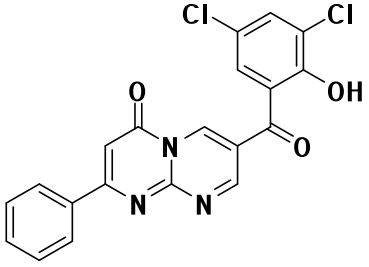
Overnight (stationary-phase) cultures of *S. aureus* strains (EMRSA-15 and EMRSA-16) were grown in Luria Broth at 37 °C in 25 ml Universal tubes mounted in a rolling drum. These cultures were used to inoculate aliquots (200 µl) of LB dispensed into the central 60 wells of a 96-well polystyrene microtitre plate. The outside wells were filled with water. Each well was also supplemented with the compound of interest at the indicated concentration. Control wells were supplemented with DMSO alone. The plates were then incubated at 37 °C to ensure good aeration of the cultures. Each hour, the plates were removed for spectrophotometric determination of the cell density (absorbance at 595 nm). After this, the plates were returned as soon as possible to the orbital shaker to ensure continuous aeration and growth. In this way, growth curves could be constructed for each strain in the presence of the appropriate compounds. This allowed the assessment of the effects of these compounds on (i) the growth rate during exponential-phase growth, and (ii) the final optical density achieved by each culture

during the stationary-phase. All experiments were performed independently in triplicate.

1.7 Compounds displaying a marked antibacterial effect (MAE).

Total list of compounds synthesized during original diversity-oriented synthesis (DOS) campaign and analogue studies which displayed a marked antibacterial effect (MAE, complete inhibition of cell growth) against EMRSA-15 and/or EMRSA-16 at a compound concentration of 100 μM or below.

Number	Structure	MRSA 15 MAE / μM	MRSA 16 MAE / μM
19		100.0	100.0
20		100.0	100.0
21		50.0	50.0
22		100.0	> 100.0

16		100.0	100.0
17		100.0	100.0
23		> 100.0	100.0
24		50.0	50.0
25		100.0	> 100.0
26		50.0	50.0

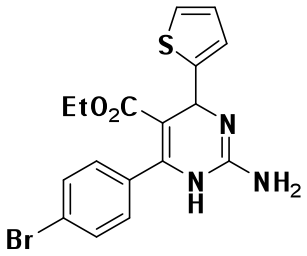
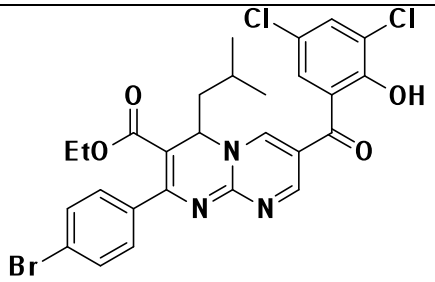
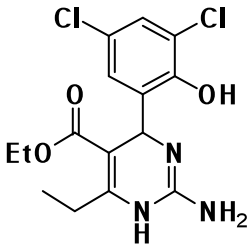
27		100.0	100.0
28		50	50
Emmacin		25	50

Table 1: Compounds displaying a MAE against EMRSA-15 and/or EMRSA-16 at concentrations of 100 μM or less.

1.8 Dihydrofolate reductase assay

1.8.1 Background

- Dihydrofolate reductase (DHFR) catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, using NADPH as a cofactor. This is required for the biosynthesis of nucleotidic bases of DNA. Thus inhibition of DHFR blocks DNA synthesis, thereby arresting cell growth.

- The effect of emmacin on EMRSA-16 DHFR (DfrB) activity was determined using a DHFR assay kit (Sigma Aldrich, Product Code CS0340). Assay was performed according to the manufacturer's instruction.

- The assay is based on the ability of DHFR to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid to terahydrofolic acid. The reaction monitors the decrease in absorbance at 340 nm as the dihydrofolic acid is consumed. At pH 7.5 the reaction goes to completion in 2.5 minutes using human DHFR enzyme. However, the bacterial DHFR enzyme requires the reaction to be carried out for longer periods of time (40 minutes at 37°C). This lower reaction rate has previously been reported for bacterial DHFRs.[4-6]

- Genomic DNA from strain MRSA-16 was used as a template to PCR-amplify the *dfrB* gene. PCR amplification was accomplished using taq DNA polymerase and primers 5'CCCCGGATCCACTTTATCCATTCTAGTTGC3' and 5'CCCCAAGCTTATTTTTTACGAATTAAATG3'. The PCR product was gel-purified and digested with *Bam*HI and *Hin*dIII enzymes. The digested PCR product was column-purified and introduced into pQE80 (Qiagen) that had been cut with the same restriction enzymes. The resulting construct was introduced into *E. coli* DH5α and both strands of the plasmid insert were sequenced to ensure that no errors had been introduced during the cloning. The pQE80 vector introduces an N-terminal hexa-histidine tag into the encoded protein, so Ni-NTA chromatography was used to purify the DfrB protein. Briefly, 1L of cells were grown up in LB medium (containing 50 μg/mL carbenicillin) at 37 °C with good aeration. Expression of the cloned gene was induced at OD = 0.5 with 1mM

IPTG. After 2 further hours of growth, the culture was harvested and the cells were resuspended in phosphate-buffered saline solution containing 10mM imidazole (PBSI buffer). The cells were lysed on ice by sonication and the lysate was clarified by centrifugation (8000 x g, 20 min, 4 °C). The cleared lysate was loaded onto a 2mL Ni-NTA column and washed overnight with 500 mL PBSI. The bound protein was eluted in PBSI supplemented with 290mM imidazole. The protein was quantified according to its A_{280} and confirmed to be >98% pure by SDS-PAGE analysis. Stored in aliquots frozen at -20 °C until use. The overall yield of DfrB was 35 mg/L of culture.

- Inhibitor (emmacin)-compound was suspended in DHFR assay buffer (Sigma) and stored at room temperature.

1.8.2 Results

1.8.2.1 Activity of sample enzyme

- Calculated by method outlined in manufacturers assay instructions

Activity of $DfrB_{EMRSA16}$ calculated as 0.0154 ± 0.008 µmol/min/mg

1.8.2.2 Concentration of emmacin required to inhibit the activity of $DfrB_{EMRSA16}$ by 50% *in vitro* (IC_{50} value)

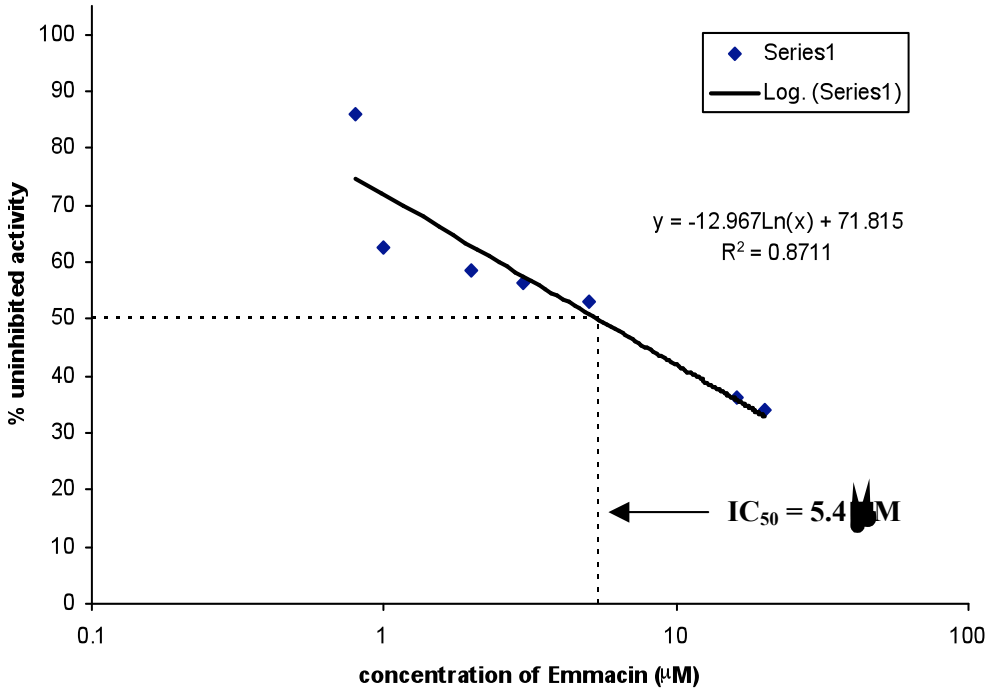


Figure 2: Variation in the % inhibition activity (rate) of the $DfrB_{EMRSA16}$ enzyme with the concentration of emmacin used. The control specific activity of the enzyme measured in the absence of emmacin in the assay mix was taken as 100% activity.

Conclusion: This data gives an IC_{50} of 5.4 µM for the inhibition of $DfrB_{EMRSA16}$ by emmacin.

1.8.3 Determining the method of inhibition of $DfrB_{EMRSA16}$ by emmacin

1.8.3.1 Varying the concentration of the substrate with inhibitor (emmacin) present

Concentration of substrate (μM)	Specific Activity of $DfrB_{EMRSA16}$ (μmol/min/mg)	Error (μmol/min/mg)
25	0.0045	±0.00084
12.5	0.0030	± 0.00037

Table 2: Variation in the specific activity of $DfrB_{EMRSA16}$ enzyme with concentration of substrate (dihydrofolic acid) in the presence of Emmacin.

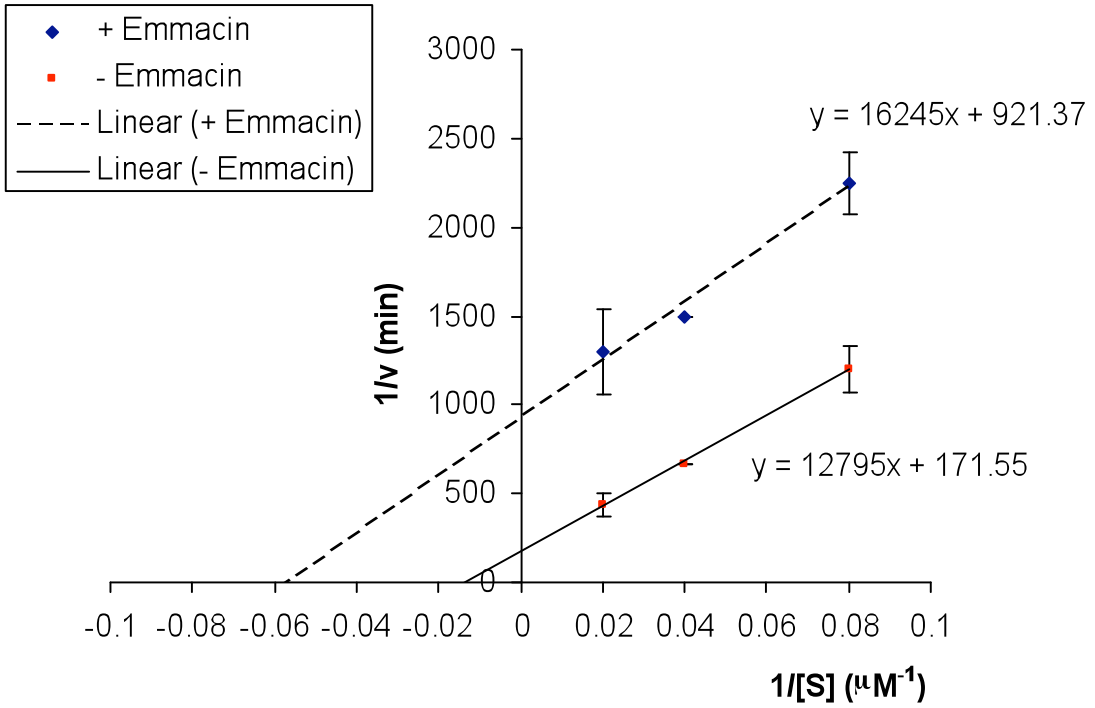
1.8.3.2 Varying the concentration of substrate with no inhibitor (emmacin) present

Concentration of substrate (μM)	Specific Activity of $DfrB_{EMRSA16}$ (μmol/min/mg)	Error (μmol/min/mg)
25	0.0102	± 0.107
12.5	0.0056	±0.00022

Table 3: Variation in the specific activity of $DfrB_{EMRSA16}$ enzyme with concentration of substrate (dihydrofolic acid) in the absence of emmacin.

1.8.3.3 Lineweaver-Burk Plot

- Activity converted to rate (velocity). Rate (velocity) calculated as the corrected change in optical density of the sample (min^{-1}).



Reaction parameters			
V_{MAX} (μMmin^{-1})		K_M (μM)	
+ Emmacin	**– Emmacin**	**+ Emmacin**	**– Emmacin**
0.0011	0.0058	17.87	74.21

Figure 4: Lineweaver-Burk plot of enzymatic reaction parameters for the conversion of dihydrofolic acid to terahydrofolic acid by $DfrB_{EMRSA16}$; + Emmacin indicates reaction in the presence of Emmacin; - Emmacin indicates reaction in the absence of Emmacin; v = reaction velocity (rate); [S] = substrate concentration; V_{MAX} = maximum velocity of enzyme; K_M = substrate concentration required for enzyme to reach half maximum velocity.

Conclusion: K_M and V_{MAX} decrease in the presence of emmacin. Overall this data implies that emmacin is an uncompetitive reversible inhibitor of $DfrB_{EMRSA16}$.[7]

1.9 References

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1.10 Appendix 1