Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Construction of six membered fused *N*-heterocyclic ring *via* a new 3-component reaction: Synthesis of (pyrazolo)pyrimidines/pyridines

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Chemistry

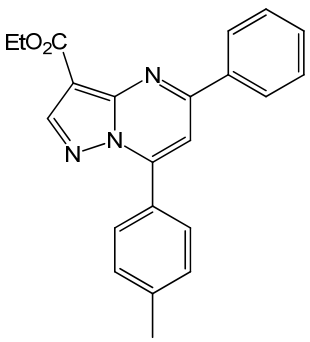
General methods

Unless stated otherwise, reactions were monitored by thin layer chromatography (TLC) on silica gel plates (60 F_{254}), visualizing with ultraviolet light or iodine spray. Column chromatography was performed on silica gel (60-120 mesh) using distilled petroleum ether and ethyl acetate. ^{1}H and ^{13}C NMR spectra were determined in $CDCl_3$/DMSO-d_6 solution using 400 and 100 MHz spectrometers, respectively. Proton chemical shifts (δ) are relative to tetramethylsilane (TMS, δ = 0.0) as internal standard and expressed in parts per million. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), and m (multiplet) as well as b (broad). Coupling constants *(J)* are given in hertz. Infrared spectra were recorded on a FTIR spectrometer. Melting points were determined by using a Buchi melting point B-540 apparatus. MS spectra were obtained on a mass spectrometer. HRMS was determined using waters LCT premier XETOF ARE-047 apparatus.

General procedure for the preparation of compound 4 (and 5)

A mixture of 3-amino-1*H*-pyrazole-4-carboxylic acid ethyl ester (1.0 mmol), an appropriate aldehyde (1 mmol) and a terminal alkyne (1 mmol) in acetic acid (5 mL) was stirred at 25 °C for 10 min. To this clear solution was added triflic acid (10 mol %) and the mixture was heated to 100-110°C. The reaction mixture was then stirred at same temperature for 2 h. After completion of the reaction the mixture was cooled to room temperature, poured into ethyl acetate (25 mL) and washed with brine solution (2 x 15 mL) followed by 10% $NaHCO_3$ solution (2 x 15 mL). The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated. The residue isolated was purified by column chromatography using petroleum ether-EtOAc to give the desired product.

Ethyl 5-phenyl-7-(*p*-tolyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4a)



Light yellow solid, mp 162-163 °C.

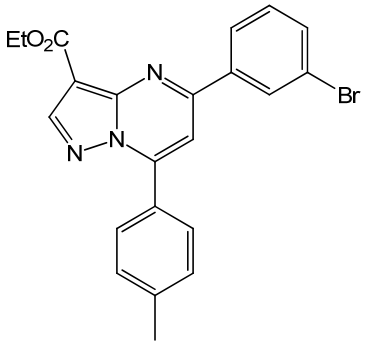
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.59 (s, 1H, CH), 8.29-8.26 (m, 2H, CH), 7.96 (d, *J*=8.3 Hz, 2H, CH), 7.55-7.52 (m, 4H, CH), 7.43 (d, *J*=8.3 Hz, 2H, CH), 4.47 (q, *J*=6.8 Hz, 2H, OCH_2), 2.48 (s, 3H, CH_3), 1.50 (t, *J*=6.8 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.9, 158.9, 148.9, 148.0, 147.7, 142.0, 136.7, 131.0, 129.5, 129.4, 128.9, 127.8, 127.7, 106.0, 102.9, 60.2, 21.6, 14.5

IR (KBr): 2975, 1684, 1611, 1558 cm^{-1}

HRMS (ESI): calcd for $C_{22}H_{20}N_3O_2$ $(M+H)^+$ 358.1556, found 358.1541

Ethyl 5-(3-bromophenyl)-7-(p-tolyl)pyrazolo[1,5-a]pyrimidine-3-carboxylate (4b)



Light yellow solid, mp 142-144 °C.

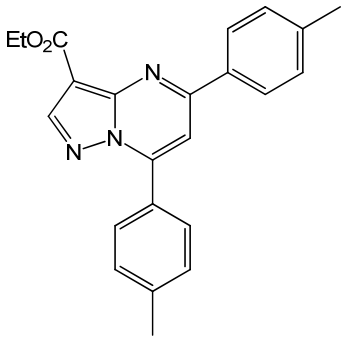
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.61 (s, 1H, CH), 8.42 (t, *J* = 2.0 Hz, 1H, CH), 8.21 (d, *J*=7.9 Hz, 1H, CH), 7.96 (d, *J*=7.9 Hz, 2H, CH), 7.67 (d, *J*=7.9 Hz, 1H, CH), 7.47 (s, 1H, CH), 7.43 (d, *J*=7.9 Hz, 2H, CH), 7.41-7.39 (m, 1H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 2.48 (s, 3H, CH_3), 1.49 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.7, 157.2, 148.7, 148.3, 147.9, 142.2, 138.7, 133.8, 130.6, 130.4, 129.5, 129.4, 127.6, 126.2, 123.2, 105.7, 103.2, 60.3, 21.6, 14.5

IR (KBr): 2979, 1691, 1613, 1557 cm^{-1}

HRMS (ESI): calcd for $C_{22}H_{19}N_3O_2Br$ $(M+H)^+$ 436.0661, found 436.0651

Ethyl 5,7-di-*p*-tolylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4c)



Light yellow solid, mp 129-131°C.

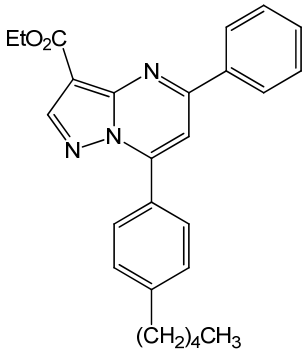
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.57 (s, 1H, CH), 8.19 (d, *J*=7.8 Hz, 2H, CH), 7.94 (d, *J*=7.8 Hz, 2H, CH), 7.49 (s, 1H, CH), 7.42 (d, *J*=7.8 Hz, 2H, CH), 7.35 (d, *J*=7.8 Hz, 2H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 2.48 (s, 3H, CH_3), 2.45 (s, 3H, CH_3),1.48 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.9, 158.9, 149.0, 147.9, 147.6, 141.9, 141.6, 133.9, 129.7, 129.5, 129.4, 127.9, 127.6, 105.8, 102.7, 60.1, 29.7, 21.6, 21.5, 14.5

IR (KBr): 2974, 1693, 1610, 1560 cm^{-1}

HRMS (ESI): calcd for $C_{23}H_{22}N_3O_2$ $(M+H)^+$ 372.1712, found 372.1700

Ethyl 7-(4-pentylphenyl)-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4d)



Light yellow solid, mp 81-83 °C.

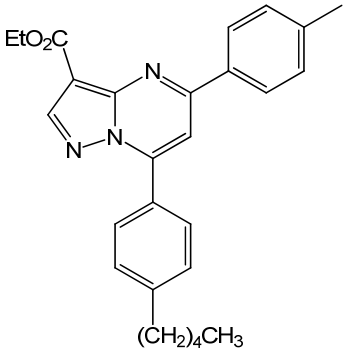
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.60 (s, 1H, CH), 8.29-8.27 (m, 2H, CH), 7.98 (d, *J*=8.3 Hz, 2H, CH), 7.55-7.53 (m, 4H, CH), 7.43 (d, *J*=8.3 Hz, 2H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 2.73 (t, *J*=7.3 Hz, 2H, CH_2), 1.70 (p, *J*=7.3 Hz, 2H, CH_2), 1.48 (t, *J*=7.3 Hz, 3H, CH_3), 1.39-1.36 (m, 4H, CH_2), 0.92 (t, *J*=7.3 Hz, 3H, CH_3),

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.7, 158.8, 148.9, 147.9, 147.6, 146.8, 136.5, 130.9, 129.3, 129.0, 128.8, 128.7, 128.6, 127.9, 127.5, 105.9, 102.8, 60.1, 35.8, 35.7, 31.4, 30.7, 22.4, 14.4, 13.9

IR (KBr): 2929, 1691, 1611, 1558 cm^{-1}

HRMS (ESI): calcd for $C_{26}H_{28}N_3O_2$ $(M+H)^+$ 414.2182, found 414.2174

Ethyl 7-(4-pentylphenyl)-5-(*p*-tolyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4e)



Yellow solid, mp 80-82 °C.

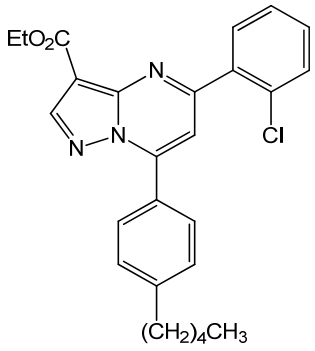
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.58 (s, 1H, CH), 8.19 (d, *J*=8.3 Hz, 2H, CH), 7.97 (d, *J*=8.3 Hz, 2H, CH), 7.50 (s, 1H, CH), 7.42 (d, *J*=8.3 Hz, 2H, CH), 7.35 (d, *J*=8.3 Hz, 2H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 2.72 (t, *J*=7.8 Hz, 2H, CH_2), 2.45 (s, 3H, CH_3), 1.69 (p, *J*=7.8 Hz, 2H, CH_2), 1.48 (t, *J*=7.3 Hz, 3H, CH_3), 1.39-1.35 (m, 4H, CH_2), 0.92 (t, *J*=7.3 Hz, 3H, CH_3),

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.9, 158.9, 149.0, 147.8, 147.6, 146.8, 141.5, 133.8, 129.7, 129.3, 128.8, 128.0, 127.5, 105.8, 102.6, 60.1, 35.9, 31.4, 30.8, 22.5, 21.4, 14.5, 13.9

IR (KBr): 2972, 1685, 1612, 1555 cm^{-1}

HRMS (ESI): calcd for $C_{27}H_{30}N_3O_2$ $(M+H)^+$ 428.2338, found 428.2336

Ethyl 5-(2-chlorophenyl)-7-(4-pentylphenyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4f)



Light yellow solid, mp 74-76 °C.

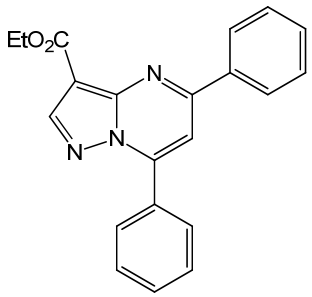
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.63 (s, 1H, CH), 8.00 (d, *J*=8.3 Hz, 2H, CH), 7.94-7.92 (m, 1H, CH), 7.56 (s, 1H, CH), 7.52-7.50 (m, 1H, CH), 7.45-7.40 (m, 4H, CH), 4.45 (q, *J*=6.8 Hz, 2H, OCH_2), 2.72 (t, *J*=7.8 Hz, 2H, CH_2), 1.69 (p, *J*=7.3 Hz, 2H, CH_2), 1.43 (t, *J*=7.3 Hz, 3H, CH_3), 1.39-1.34 (m, 4H, CH_2), 0.91 (t, *J*=7.3 Hz, 3H, CH_3)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.6, 159.1, 148.8, 147.5, 147.0, 146.8, 137.1, 132.3, 132.1, 131.0, 130.3, 129.4, 128.8, 127.6, 127.3, 110.5, 103.2, 60.2, 35.9, 31.4, 30.8, 22.4, 14.4, 13.9

IR (KBr): 2956, 1715, 1608, 1557 cm^{-1}

HRMS (ESI): calcd for $C_{26}H_{27}N_3O_2Cl$ $(M+H)^+$ 448.1792, found 448.1773

Ethyl 5,7-diphenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4g)



Yellow solid, mp 79-81°C.

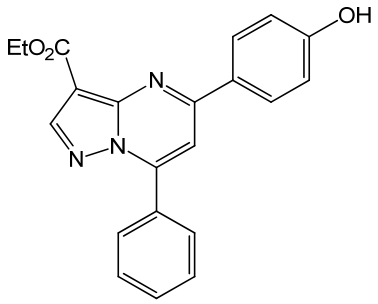
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.60 (s, 1H, CH), 8.29-8.27 (m, 2H, CH), 8.05-8.02 (m, 2H, CH), 7.63-7.61 (m, 3H, CH), 7.55-7.52 (m, 3H, CH), 7.53 (s, 1H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 1.48 (t, *J*=7.3 Hz, 3H, CH_3)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.8, 158.9, 148.9, 147.8, 147.7, 136.5, 131.3, 131.1, 130.7, 129.4, 128.9, 128.8, 127.6, 106.3, 103.0, 60.2, 14.5

IR (KBr): 2982, 1687, 1613, 1554 cm^{-1}

HRMS (ESI): calcd for $C_{21}H_{18}N_3O_2$ $(M+H)^+$ 344.1399, found 344.1382

Ethyl 5-(4-hydroxyphenyl)-7-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4h)



Yellow solid, mp 196-198 °C.

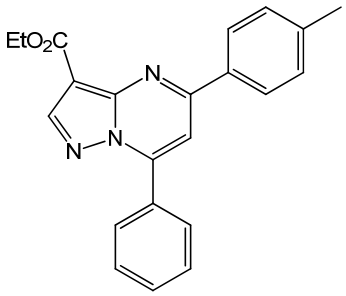
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.55 (s, 1H, CH), 8.23 (d, *J*=7.8 Hz, 2H, CH), 8.02-8.00 (m, 2H, CH), 7.62-7.60 (m, 3H, CH), 7.47 (s, 1H, CH), 7.14 (d, *J*=7.8 Hz, 2H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 1.48 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 163.7, 159.7, 159.4, 149.4, 147.7, 147.5, 131.3, 130.8, 129.6, 129.4, 128.8, 128.5, 116.2, 106.3, 101.9, 60.6, 14.5

IR (KBr): 3243, 2982, 1686, 1605 cm^{-1}

HRMS (ESI): calcd for $C_{21}H_{18}N_3O_3$ $(M+H)^+$ 360.1348, found 360.1333

Ethyl 7-phenyl-5-(*p*-tolyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4i)



Yellow solid, mp 120-122°C.

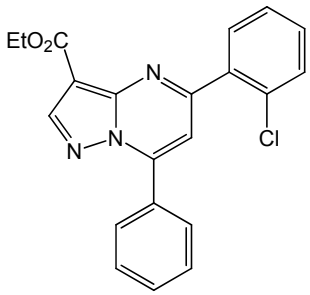
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.58 (s, 1H, CH), 8.20 (d, *J*=8.3 Hz, 2H, CH), 8.04-8.01 (m, 2H, CH), 7.63-7.60 (m, 3H, CH), 7.50 (s, 1H, CH), 7.35 (d, *J*=8.3 Hz, 2H, CH), 4.47 (q, *J*=7.3 Hz, 2H, OCH_2), 2.45 (s, 3H, CH_3), 1.48 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.8, 158.9, 148.9, 147.6, 141.6, 133.7, 131.2, 130.7, 129.6, 129.4, 128.7, 127.5, 106.1, 102.8, 60.1, 21.4, 14.5

IR (KBr): 2982, 1686, 1606, 1556 cm^{-1}

HRMS (ESI): calcd for $C_{22}H_{20}N_3O_2$ $(M+H)^+$ 358.1556, found 358.1543

Ethyl 5-(2-chlorophenyl)-7-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4j)



Light yellow solid, mp 156-158 °C.

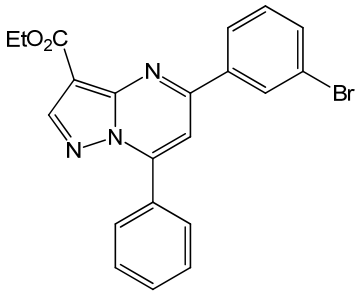
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.63 (s, 1H, CH), 8.07-8.06 (m, 2H, CH), 7.94-7.92 (m, 1H, CH), 7.61-7.51 (m, 4H, CH), 7.57 (s, 1H, CH), 7.45-7.43 (m, 2H, CH), 4.45 (q, *J*=7.3 Hz, 2H, OCH_2), 1.43 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.6, 159.2, 148.8, 147.6, 146.7, 137.1, 132.4, 132.2, 131.5, 131.1, 130.5, 130.3, 129.5, 128.8, 127.4, 110.9, 103.4, 60.3, 14.5

IR (KBr): 2981, 1705, 1607, 1554 cm^{-1}

HRMS (ESI): calcd for $C_{21}H_{17}N_3O_2Cl$ $(M+H)^+$ 378.1009, found 378.0997

Ethyl 5-(3-bromophenyl)-7-phenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4k)



Yellow solid, mp 133-135 °C.

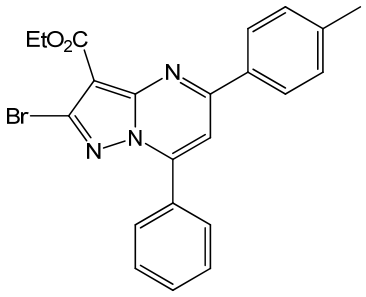
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.62 (s, 1H, CH), 8.43 (t, *J* = 2.0 Hz, 1H, CH), 8.22 (d, *J*=7.9 Hz, 1H, CH), 8.06-8.03 (m, 2H, CH), 7.67-7.61 (m, 4H, CH), 7.49 (s, 1H, CH), 7.44-7.40 (m, 1H, CH), 4.48 (q, *J*=7.3 Hz, 2H, OCH_2), 1.49 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.7, 157.3, 148.7, 148.2, 148.0, 138.6, 134.0, 131.6, 130.6, 130.5, 129.5, 128.9, 126.2, 123.3, 106.1, 103.3, 60.3, 14.5

IR (KBr): 2975, 1703, 1612, 1559 cm^{-1}

HRMS (ESI): calcd for $C_{21}H_{17}N_3O_2Br$ $(M+H)^+$ 422.0504, found 422.0485

Ethyl 2-bromo-7-phenyl-5-(*p*-tolyl)pyrazolo[1,5-*a*]pyrimidine-3-carboxylate (4l)



Light yellow solid, mp 160-162 °C.

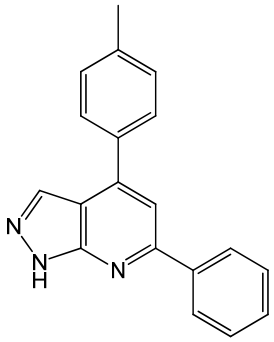
^{1}H NMR ($CDCl_3$, 400 MHz): 8.17 (d, *J*=7.8 Hz, 2H, CH), 8.04-8.01 (m, 2H, CH), 7.61-7.59 (m, 3H, CH), 7.50 (s, 1H, CH), 7.35 (d, *J*=7.8 Hz, 2H, CH), 4.51 (q, *J*=7.3 Hz, 2H, OCH_2), 2.45 (s, 3H, CH_3), 1.52 (t, *J*=7.3 Hz, 3H, CH_3).

^{13}C NMR ($CDCl_3$, 100 MHz): δ 161.9, 159.1, 149.6, 146.9, 141.9, 137.7, 133.5, 131.5, 130.1, 129.7, 129.5, 128.8, 127.5, 106.4, 102.1, 60.4, 21.5, 14.4

IR (KBr): 2977, 1704, 1605, 1550 cm^{-1}

HRMS (ESI): calcd for $C_{22}H_{19}N_3O_2Br$ $(M+H)^+$ 436.0661, found 436.0672

6-Phenyl-4-(*p*-tolyl)-1*H*-pyrazolo[3,4-*b*]pyridine (5a)



Off-white solid, mp 196-198 °C.

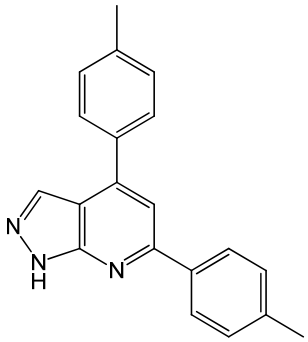
^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.22 (bs, 1H, NH), 8.26 (s, 1H, CH), 8.14 (d, *J* = 7.9 Hz, 2H, CH), 7.75-7.71 (m, 3H, CH), 7.56-7.48 (m, 3H, CH), 7.41 (d, *J*=7.9 Hz, 2H, CH), 2.48 (s, 3H, CH_3)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 157.8, 153.0, 144.8, 139.6, 139.5, 134.7, 133.8, 129.9, 129.5, 128.9, 128.3, 127.7, 113.7, 110.5, 21.4

IR (KBr): 3142, 3033, 2851, 1596 cm^{-1}

HRMS (ESI): calcd for $C_{19}H_{16}N_3$ $(M+H)^+$ 286.1344, found 286.1335

4,6-Di-*p*-tolyl-1*H*-pyrazolo[3,4-*b*]pyridine (5b)



Light yellow solid, mp 229-231 °C.

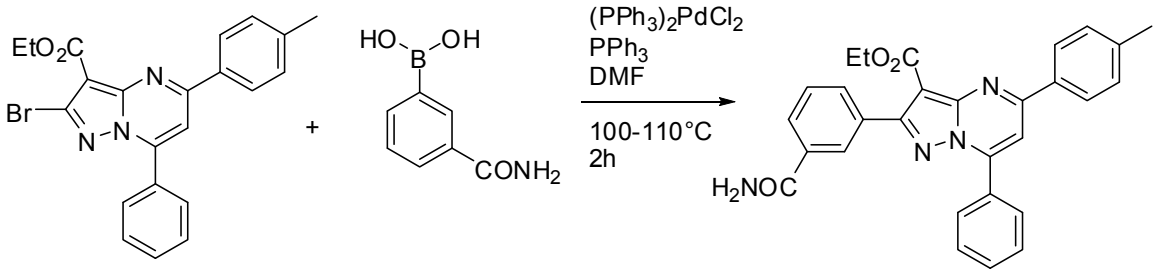
^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.34 (bs, 1H, NH), 8.25 (s, 1H, CH), 8.05 (d, *J*=8.3 Hz, 2H, CH), 7.74 (d, *J*=7.8 Hz, 2H, CH), 7.69 (s, 1H, CH), 7.41 (d, *J*=7.8 Hz, 2H, CH), 7.36 (d, *J*=8.3 Hz, 2H, CH), 2.48 (s, 3H, CH_3), 2.45 (s, 3H, CH_3)

^{13}C NMR (DMSO-d_6, 100 MHz): δ 156.0, 152.9, 143.2, 139.1, 139.0, 136.2, 134.2, 132.5, 129.8, 129.4, 128.3, 127.2, 111.8, 111.3, 20.9, 20.8

IR (KBr): 3144, 3024, 2857, 1595 cm^{-1}

HRMS (ESI): calcd for $C_{20}H_{18}N_3$ $(M+H)^+$ 300.1501, found 300.1490

Suzuki coupling of 4l with 3-carbamoylphenylboronic acid



4l

A mixture of **4l** (1 mmol), PPh_3 (0.15 mmol), $(PPh_3)_2PdCl_2$ (2 mol%) and sodium carbonate (4 mmol) in DMF (5 mL) was stirred at 25 °C for 30 mins. To this mixture was added boronic acid (1 mmol) at room temperature. The reaction mixture was then stirred at 100-110°C for 2 h, cooled to room temperature. The reaction mixture was poured into DCM (25 mL) and washed with brine

solution (3 x 15 mL). The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated. The residue was triturated with ethyl acetate; the solid precipitate was filtered and washed with diisopropyl ether to give the desired product as light brown solid (yield 92%).

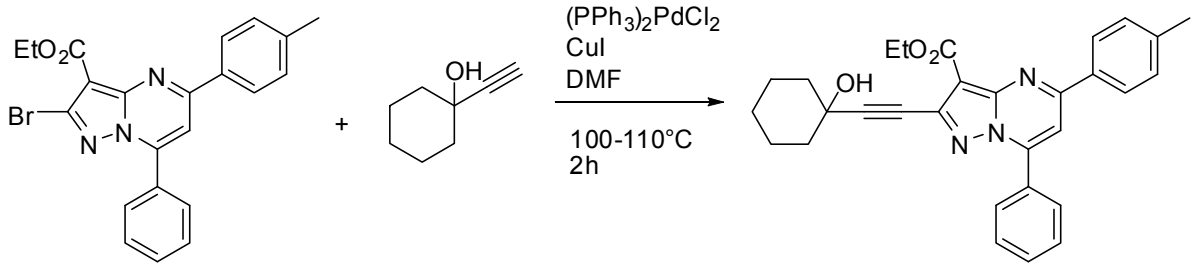
^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.28 (s, 1H, CH), 8.20 (d, *J*=8.3 Hz, 2H, CH), 8.11-8.08 (m, 2H, CH), 8.00-7.92 (m, 2H, CH), 7.61-7.51 (m, 5H, CH), 7.37 (d, *J*=8.3 Hz, 2H, CH), 6.24 (bs, 1H, NH), 5.62 (bs, 1H, NH), 4.47 (q, *J*=7.3Hz, 2H, OCH_2), 2.46 (s, 3H, CH_3), 1.40 (t, *J*=7.3 Hz, 3H, CH_3)

^{13}C NMR (DMSO-d_6, 100MHz): δ 167.5, 162.2, 157.8, 156.8, 149.6, 146.6, 141.3, 133.9, 133.4, 132.7, 132.4, 131.2, 130.3, 129.9, 129.5, 128.8, 128.4, 127.9, 127.7, 127.6, 106.7, 99.5, 59.6, 21.0, 14.0

IR (KBr): 3477, 3189, 2977, 1681cm^{-1}

HRMS (ESI): calcd for $C_{29}H_{25}N_4O_3$ $(M+H)^+$ 477.1927, found 477.1924

Sonogashira coupling of 4l with 1-ethynylcyclohexanol



4l

A mixture of **4l** (1 mmol), $(PPh_3)_2PdCl_2$ (2 mol%), CuI (0.02 mmol) and triethylamine (4 mmol) in DMF (5 mL) was stirred at 25 °C for 30 mins. To this mixture was added alkyne (1.2 mmol) slowly with stirring. The reaction mixture was then stirred at 100-110°C for 2 h, cooled to room temperature. The reaction mixture was poured into DCM (25 mL) and washed with brine solution (3 x 15 mL). The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography using Petroleum ether-EtOAc to give the desired product as a light yellow solid (yield 75%).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.18 (d, *J*=8.3 Hz, 2H, CH), 8.05-8.02 (m, 2H, CH), 7.60-7.59 (m, 3H, CH), 7.52 (s, 1H, CH), 7.35 (d, *J*=8.3 Hz, 2H, CH), 4.50 (q, *J*=7.3 Hz, 2H, OCH_2), 2.48 (bs,

1H, OH), 2.45 (s, 3H, CH_3), 2.09 (t, *J*=7.3 Hz, 2H, CH_2), 1.76-1.57 (m, 8H, CH_2), 1.50 (t, *J*=7.3 Hz, 3H, CH_3)

^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.3, 158.9, 149.1, 146.9, 141.7, 141.4, 133.6, 131.4, 130.4, 129.7, 129.5, 128.7, 127.5, 106.8, 104.1, 99.8, 76.5, 69.0, 60.3, 39.5, 25.2, 23.0, 21.5, 14.5

IR (KBr): 3411, 2926, 2230, 1678 cm^{-1}

HRMS (ESI): calcd for $C_{30}H_{30}N_3O_3$ $(M+H)^+$ 480.2287, found 480.2276

Pharmacology

Materials and Methods

Cells and Reagents: HEK 293 and Sf9 cells were obtained from ATCC (Washington D.C., USA). HEK 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen Inc., San Diego, CA, USA). Sf9 cells were routinely maintained in Grace's supplemented medium (Invitrogen) with 10% FBS. RAW 264.7 cells (murine macrophage cell line) were obtained from ATCC and routinely cultured in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen Inc.). cAMP was purchased from SISCO Research Laboratories (Mumbai, India). PDElight HTS cAMP phosphodiesterase assay kit was procured from Lonza (Basel, Switzerland).

PDE4B protein production and purification

PDE4B cDNA was sub-cloned into pFAST Bac HTB vector (Invitrogen) and transformed into DH10Bac (Invitrogen) competent cells. Recombinant bacmids were tested for integration by PCR analysis. Sf9 cells were transfected with bacmid using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Subsequently, P3 viral titer was amplified, cells were infected and 48 h post infection cells were lysed in lysis buffer (50 mM Tris-HCl pH 8.5, 10 mM 2-Mercaptoethanol, 1 % protease inhibitor cocktail (Roche), 1 % NP40). Recombinant His-tagged PDE4B protein was purified as previously described elsewhere (Wang et al., 1997). Briefly, lysate was centrifuged at 10,000 rpm for 10 min at 4˚C and supernatant was collected. Supernatant was mixed with Ni-NTA resin (GE Life Sciences) in a ratio of 4:1 (v/v) and equilibrated with binding buffer (20 mM Tris-HCl pH 8.0, 500 mM-KCl, 5 mM imidazole, 10 mM 2-mercaptoethanol and 10 % glycerol) in a ratio of 2:1 (v/v) and mixed gently on rotary shaker for 1 hour at 4˚C. After incubation, lysate-Ni-NTA mixture was centrifuged at 4,500 rpm for 5 min at 4˚C and the supernatant was collected as the flow-through fraction. Resin was washed twice with wash buffer

(20 mM Tris-HCl pH 8.5, 1 M KCl, 10 mM 2-Mercaptoethanol and 10% glycerol). Protein was eluted sequentially twice using elution buffers (Buffer I: 20 mM Tris-HCl pH 8.5, 100 mM KCl, 250 mM imidazole, 10 mM 2-mercaptoethanol, 10% glycerol, Buffer II: 20 mM Tris-HCl pH 8.5, 100 mM KCl, 500 mM imidazole, 10 mM 2-mercaptoethanol, 10% glycerol). Eluates were collected in four fractions and analyzed by SDS-PAGE. Eluates containing PDE4B protein were pooled and stored at -80˚C in 50% glycerol until further use.

PDE4B enzymatic assay

The inhibition of PDE4B enzyme was measured using PDElight HTS cAMP phosphodiesterase assay kit (Lonza) according to manufacturer's recommendations. Briefly, 10 ng of PDE4B enzyme was pre-incubated either with DMSO (vehicle control) or compound for 15 min before incubation with the substrate cAMP (5 µM) for 1 h. The reaction was halted with stop solution followed by incubation with detection reagent for 10 minutes in dark. Luminescence values (RLUs) were measured by a Multilabel plate reader (Perklin Elmer 1420 Multilabel counter). The percentage of inhibition was calculated using the following formula:

$$\%\ inhibition = \frac{(RLU\ of\ vehicle\ control - RLU\ of\ inhibitor)}{RLU\ of\ vehicle\ control} X\ 100$$

Docking study

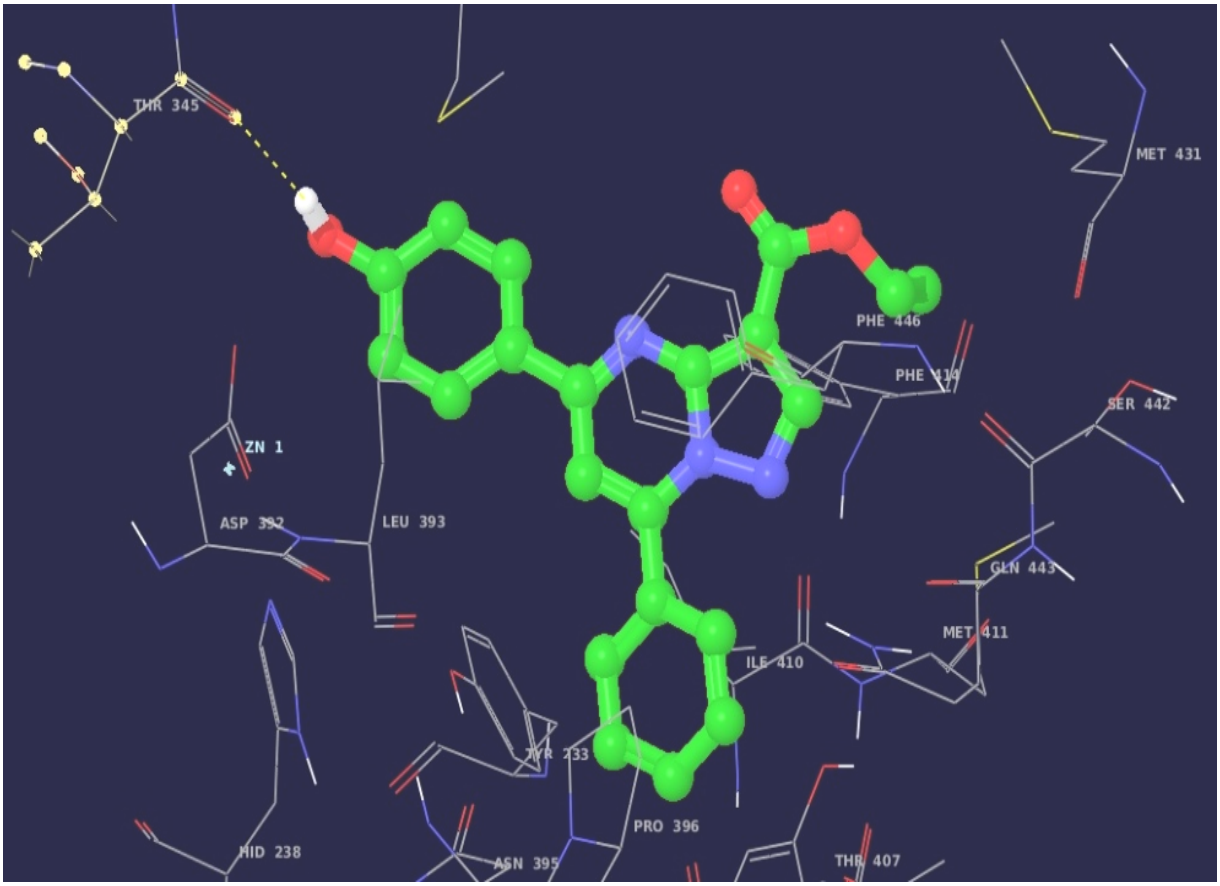
The molecular docking study was performed using XP Glide application of schrodinger software with MASTERO interface 9.2

Molecules were docked in the PDE4B protein and their respective glide scores and interactions were observed.

Procedure: In the present study we have performed the energy minimization and conformational search with the MACROMODEL application in the Schrodinger package. The study molecule was energy minimized for flexibility and then used for conformational search. We used OPLS_2005 force field and water as implicit solvent. We have followed the PRCG (Polak-Ribier conjugate gradient) method of minimization with 500 iterations with a threshold gradient on 0.05kJ/mol. The

conformational search was based on Montecarlo multiple minimum torsional sampling .The ligand was then finally prepared with LIGPREP application.

The PDE4B (PDB.ID-3D3P) protein crystal structure was retrieved from the protein data bank and it was refined with the PROTEIN PREPERATION WIZARD application in which the hydrogen's were added and the missing side chains and loops were filled with PRIME application. The water molecules were observed within the distance of 5A and other water molecules beyond 5A from het (heteroatom) groups were deleted. Finally the protein is then optimized and minimized with impref using OPLS_2005 force filed. GRID based Flexible Docking was done in the present study.



The following GLIDE scores and other parameters were obtained after docking with PDE4B protein:-

Total Glide score = -10.2 K.cal/mol

Total fraction of the VdW energy in protein-ligand interaction = – 5.7K.cal/mol

Hydrophobic ensure reward in the interaction = -2.2K.cal/mol.

Electrostatic Rewards = -0.5K.cal/mol

ChemScore H-bond pair term = -0.7K.cal/mol.