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ARTICLE

An integrated flow and microwave approach to a broad spectrum protein kinase inhibitor

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The protein kinase inhibitor, CTx-0152960 (**6**, 2-((5-chloro-2-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino)-*N*-methylbenzamide), and the piperazinyl analogue, CTx-0294885 (**7**, 2-((5-chloro-2-((4-piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-*N*-methylbenzamide), were prepared using a hybrid flow and microwave approach. The use of flow chemistry approaches avoided the need for Boc-protection of piperidine in the key S_NAr coupling with 1-fluoro-4-nitrobenzene. Microwave coupling of 4-morphilinoaniline **8** and 4-(piperazine-1-yl)aniline **9** with 2-(2,5-dichloropyrimidine-4-ylamino)-*N*-methylbenzamide **10**, proved to be the most efficacious route to the target analogues **6** and **7**. This hybrid methodology reduced the number of synthetic steps, gave enhanced overall yields increased atom economy through step reduction and minimal requirement for chromatographic purification, relative to the original batch synthesis approach.

Introduction

Protein kinase inhibitors (PKIs) are currently one of the most important classes of anticancer drugs. Globally there continues to be a major drive towards the development of more selective and potent PKI-based drugs for the treatment of an expanding range of diseases.[1-5] Imatinib was the first clinically approved PKI in 2001, with 14-PKI based drugs approved for the treatment of cancer over the next decade, e.g. Gefitinib (**2**, 2002), Vemurafenib (**3**, 2011) and Ruxolitinib (**4** 2011) (Figure 1).[6]

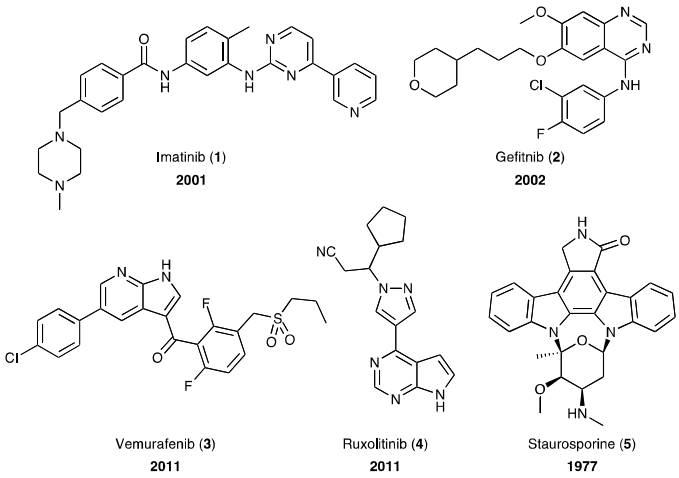

Figure 1. Chemical structures of selected clinically used PKI-based drugs: Imatinib (**1**), Gefitinib (**2**), Vemurafenib (**3**), Ruxolitinib (**4**) and the chemical structure of the protein kinases inhibitor, staurosporine (**5**) and their year of clinical approval or initial literature report.

While there are a number of selective inhibitors, the chemical biology of protein kinases has benefited greatly from the discovery and subsequent use of the broad spectrum inhibitor staurosporine (**5**).[7] Broad spectrum PKIs such as staurosporine, are of particular importance as chemical proteomics tools.[8-16] Recently 2-(5-chloro-2-(4-morpholinophenylamino)-pyrimidin-4-ylamino)-*N*-methylbenzamide, CTx-0152960 (**6**), was reported as a new member of the broad spectrum PKI family and developed as a Sepharose-supported kinase capture reagent in the identification of 235 protein kinases from a digest of human breast adenocarcinoma, MDA-MB-231, cells (Figure 2, **7**, CTx-0294885).[17,18]

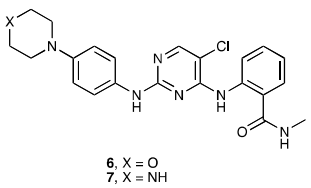

Figure 2. 2-(5-Chloro-2-(4-morpholinophenylamino)pyrimidin-4-ylamino)-*N*-methylbenzamide (**6**) and 2-(5-chloro-2-(4-(piperazine-1-yl)phenylamino)pyrimidin-4-ylamino)-*N*-methylbenzamide (**7**).

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Given the importance of the PKI families of compounds they have attracted considerable attention from the synthetic community, more recently in the application of flow chemistry approaches.[19,20] As part of an on-going program we were interested in gaining access to these probes and the parent inhibitor compound to conduct competition based kinomics experiments.[8,12,21] The synthesis of **6** and **7** was detailed by Zhang,[17,18] but as we desired access to both compounds we viewed this as an opportunity to investigate the introduction of potential efficiencies in the synthesis of both compounds via a combination of flow and microwave chemistry approaches.[22-29]

Results and discussion

On examination of **6** and **7**, we identified fragments **8**/**9** and **10** as key intermediates (Figure 2). Access to piperazine **9**, on coupling to **10**, would facilitate direct conjugation to a solid support such as Sepharose,[17] while the use of morpholine **8** would allow access to **6**. We thus set about developing the required protocols.

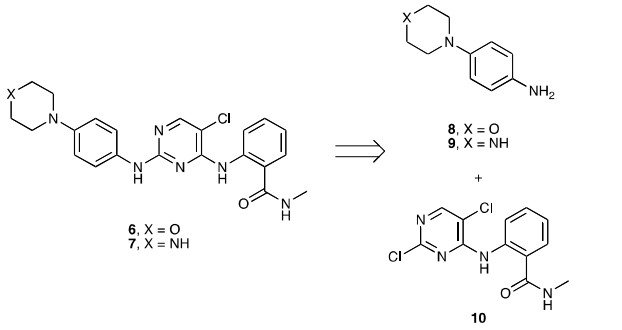
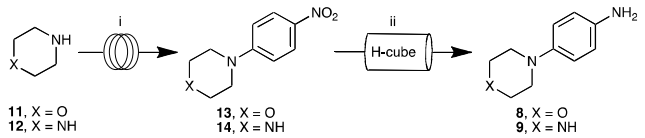


Figure 2. Identification of two key fragments for the synthesis of **6** and **7**, the anilines **8** and **9**, and the pyrimidine **10**.

Our initial investigations centred on the use of mono-boc piperazine. While commercially available, we were also interested in accessing an approach applicable across a myriad of diamine substrates. Despite the apparent simplicity of the synthesis, current batch approaches to mono-boc piperazine via Boc_2O and *tert*-butyl azido formate, typically afford a 4:1 ratio of mono- and di- Boc piperazine.[30,31] This was further confounded by the explosive nature of *tert*-butyl azido formate.[32] Despite considerable optimisation, the flow selective mono-boc protection was unsuccessful, and in keeping with a recent report by Jong and Bradley, our optimal outcome was a 88:12 ratio.[33] Despite this, we were able to carry the di-boc-piperazine through all subsequent synthetic steps with no major complications (ESI†).

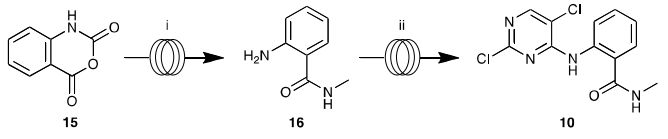
Re-evaluation of our flow strategy saw omission of the protecting group step and investigation of the direct S_NAr coupling of piperazine **12** with 1-fluoro-4-nitrobenzene. Optimisation of the flow S_NAr coupling of **12** with 1-fluoro-4-nitrobenzene at 80 ºC and 5 $mL.min^{-1}$ (Vapourtec R2+), using 5 equivalents of **12** gave exclusively 1-(4-nitrophenyl)piperazine **14** in a 95% yield (Scheme 1). The equivalent batch approaches furnish a mixture of mono- and di- adduct in keeping with our earlier efforts to mono-protect piperazine (ESI†).

Flow hydrogenation of **14**'s nitro moiety was effected cleanly as a 0.05 M MeOH solution using 10%-Pd/C at 50 °C and 50 bar H_2 at 1 $mL.min^{-1}$ to give quantitative conversion to aniline **9** (Scheme 1; ThalesNano H-CubePro®; 70 mm CatCart®).[22,25,34,35] This sequence, commencing with morpholine **11** gave the corresponding morpholino analogues **13** and **8** in excellent yields (76 and 87%, respectively) and purity (>98%) (ESI†).



Scheme 1. Reagents and Conditions: (i) Vapourtec R2+ 80 ºC, 4M **11** or **12** in DMF, 2M 1-fluoro-4-nitrobenzene in DMF, 8 bar, 5 $mL.min^{-1}$; (ii) ThalesNano H-CubePro®, 0.05 M **13** or **14** in MeOH, 10% Pd-C CatCart® (70 mm), 50 bar, 50 °C, 1.0 $mL.min^{-1}$.

Synthesis of the pyrimidine fragment **10**, commenced with the treatment of isatoic anhydride **15** with 40% (w/w) aq. $MeNH_2$ at 0.5 $mL.min^{-1}$ at 0 °C (Syrris FRX-100) to give, *N*-methylamidoaniline **16** in 79% yield (Scheme 3). The yield was identical to that obtained in the corresponding batch process. Access to pyrimidine **10** was effected by an S_NAr coupling with 2,3,5-trichloropyrimidine at 100 ºC and 0.25 $mL.min^{-1}$ (Vapourtec R2+). Pyrimidine **10** was isolated by simple filtration in 71% yield and >95% purity after concentration of the reaction stream, whereas the batch approach required longer reaction times (ESI†).[17]



Scheme 2. Reagents and conditions. (i) Syrris FRX-100: 40% w/w aq. $MeNH_2$, 0.5 $mL.min^{-1}$, 0 → 19 °C, 19 h; (ii) Vapourtec R2+: 2,3,,5-trichloropyrimidine, iPr_2NEt, *i*PrOH, 4 bar, 100 °C.

With fragments **8**/**9** and **10** in hand, we next examined the coupling to access **6** and **7**. All flow chemistry approaches failed to afford a satisfactory outcome with considerable reagent degradation observed at the temperatures and residence times required to effect an acceptable level of coupling. However, microwave assisted coupling of **9** and **10** provided **7** in a 47% yield, as a mixture of benzamide rotamers.[36] An identical microwave assisted coupling of **8** and **10** gave **6** in 51% yield (Scheme 3).

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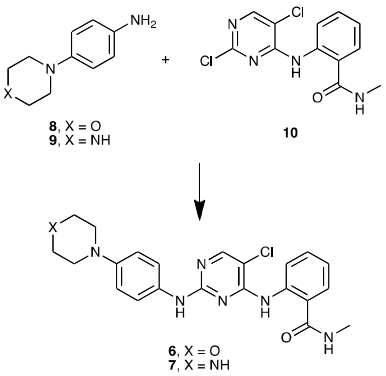

Scheme 3. Reagents and conditions. (i) *n*-BuOH, 4M HCl in dioxane (cat), 150 °C, μW 20 minutes

Conclusions

The synthesis of **13** and **14** were readily accomplished through flow S_NAr reaction either morpholine **11** or piperazine **12** with 1-flouro-4-nitrobenzene, the subsequent aryl nitro functionality of **13** and **14** were reduced to the corresponding anilines **8** and **9**, *via* flow hydrogenation. No protection of the piperazine moiety was required under these conditions. This allowed a reduction in the number of chemical steps, and thus an increase in atom economy overall. The coupling of **8**/**9** with **10** under flow conditions failed, but was smoothly affected under microwave irradiation.

Our hybrid flow and microwave assisted route provided rapid and scalable access to CTx-0152960 (**6**) and CTx-0294885 (**7**) in a five step process in overall yields of 19 and 25%, requiring essentially filtration through a short silica gel plug with minimal chromatographic requirement. This compares favourably against the initial synthesis of **7** in 6.5% yield, with multiple chromatographic requirements.[17]

Acknowledgements

Financial support was received from the Australian Research Council (ARCDP 14101565), the Australian Cancer Research Foundation and the Ramaciotti Foundation.

Experimental

All reagents were purchased from Sigma-Aldrich, Matrix Scientific or Lancaster Synthesis and were used without purification. With the exception of THF (anhydrous > 99%) obtained from Sigma-Aldrich, all solvents were re-distilled from glass prior to use.

^{1}H and ^{13}C NMR spectra were recorded on a Brüker Avance™ AMX 400 MHz spectrometer at 400 and 101 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) measured relative to the internal standards, and coupling constants (*J*) are expressed in Hertz (Hz). Mass spectra were recorded on a Shimadzu LCMS 2010 EV using a mobile phase of 1:1 acetonitrile:H_2O with 0.1% formic acid.

HPLC analysis was recorded using a Shimadzu 20A with Grace econosphere C_{18} 5μ 150 x 4.6 mm column, and GCMS analysis was carried out using a Shimadzu QP 2010 GCMS.

4-(4-Nitrophenyl)morpholine (13)

A stream of morpholine **(11)** (0.436 g, 5.0 mmol) in DMF (100 mL) and another stream of 4-fluoronitrobenzene (0.352 g, 2.5 mmol) in DMF (100 mL) was passed through a Vapourtec R2+ reaction system at 0.5 mL.min^{-1}. The instrument was fitted with two 10 mL PFA coils, maintained at 120 °C and 8 bar of pressure. The resulting product stream was taken up in DCM (200 mL) and washed with water (2 × 100 mL) and saturated NaCl (1 × 100 mL). The organic layer was then dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid (0.396 g, 76% yield).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.13 (2H, d, *J* = 9.3 Hz), 6.82 (2H, d, *J* = 9.3 Hz), 3.61 (4H, m), 3.42 (4H, m), 1.49 (9H, s); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 154.6, 154.6, 138.8, 126.0 (2C), 112.9 (2C), 80.4, 46.9 (2C), 43.9-42.1 (bs, 2C), 28.4 (3C); Mass spectrum (ESI, +ve) 308 *m/z* [(M + H)$^+$, 20%], 252 (100), 208 (7), 146 (7), 100 (13); FTIR ν_{max} (cm^{-1}): 3007, 2970, 2865, 167, 1585, 1485, 1417, 1367, 1318, 1238, 1162, 1112, 1040, 1081, 1001, 919, 830, 774, 754, 714, 691, 666, 640, 538, 500.

1-(4-Nitrophenyl)piperazine (14)

A stream of piperazine (**12**, 1.05 g, 12.1 mmol), in DMF (50 mL) solution and another stream of 4-fluoronitrobenzene (0.375 g, 2.65 mmol) in DMF (25 mL) was passed through a Vapourtec R2+ reaction system at 0.5 mL.min^{-1}. The instrument was fitted with two 10 mL PFA coils, maintained at 100 °C and 8 bar of pressure. The resulting product stream was diluted with water (50 mL), extracted with DCM (3 × 20 mL), washed with water (3 × 40 mL) and brine (1× 40 mL), dried ($MgSO_4$), filtered and then concentrated under reduced pressure to give a yellow solid (0.521 g, yield 95%), m.p. 118-122 °C. GCMS analysis of the crude reaction material revealed greater than 95% of the desired compound.

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.04 (1H, d, *J* = 8.4 Hz), 7.00 (2H, d, *J* = 8.1 Hz), 3.37 (4H, s),. ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.1, 136.6, 125.7, 112.4, 47.3, 45.1. Mass spectrum (ESI, +ve) 179 *m/z* [(M + H)$^+$, 100%] FTIR ν_{max} (cm^{-1}): 3334, 2948, 2838, 1580, 1480, 1309, 1244, 1100, 834, 753, 696, 665, 599.

4-Morphilinoaniline (8)

A solution of 4-(4-nitrophenyl)morpholine (**13**) (0.475 g, 2.28 mmol) and DMSO (3 mL) in methanol (100 mL) was pumped through a ThalesNano H-Cube® fitted with a 70 mm 10% Pd/C CatCart® at 1 mL.min^{-1}. The resulting product stream was concentrated under reduced pressure to afford a yellow oil that was then diluted with DCM (30 mL), washed with water (3 × 30mL), saturated NaCl (30 mL), dried (Mg_2SO_4), filtered and then concentrated under reduced pressure to afford a tan solid (0.350 g, 87% yield). This material was identical, in all aspects, with an authentic sample and commercially available from Sigma Aldrich.[17,18]

4-(Piperazin-1-yl)aniline (9)

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A solution of 1-(4-nitrophenyl)piperazine **14** (0.219 g, 1.06 mmol) in methanol (100 mL) was passed through a ThalesNano H-Cube Pro® using 30 mm 10% Pd-C CatCart® catalyst at 1 mL.min^{-1} at 50 °C and 50 bar of pressure. The resulting reactant stream was concentrated under reduced pressure to afford a white solid (0.187 g, 99%) that darkened and decomposed over time on standing at room temperature. This material was deemed pure by ^{1}H NMR spectroscopy and used in the subsequent step without further purification.

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.83 – 6.78 (m, 2H), 6.68 – 6.63 (m, 2H), 3.01 (tdd, *J* = 7.8, 4.0, 1.1 Hz, 8H) ; ^{13}C NMR (151 MHz, $CDCl_3$) δ 145.26, 140.25, 118.78, 116.34, 52.42, 46.46; Mass spectrum (ESI, +ve) 178 *m/z* [(M + H)$^+$, 100%], 279 (33), 222 (22); FTIR ν_{max} (cm^{-1}): 3301, 1644, 1633, 1519, 1085.

2-Amino-*N*-methylbenzamide (16)

To a flask containing isatoic anhydride (**15**) (1.86 g, 11.4 mmol), at 0 °C, methylamine (10 mL, 144.0 mmol of 40% *w*/*v* aqueous solution) at a rate of 0.25 mL.min^{-1} using a Syrris FRX-100. The mixture was warm room temperature and stirred overnight, diluted with ethyl acetate (100 mL). The aqueous layer was extracted with ethyl acetate (3 × 100 mL), and the combined organic extracts were washed with water (200 mL), brine (2 × 200 mL), dried ($MgSO_4$) and then concentrated under reduce pressure to afford the title compound (1.35 g, 79%), as an off-white solid.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.29 (1H, dd, *J* = 9.3, 1.5 Hz), 7.19 (1H, ddd, *J* = 8.6, 7.1, 1.5 Hz), 6.67 (1H, ddd, *J* = 8.1, 0.9 Hz), 6.65 (1H, ddd, *J* = 8.6, 7.2, 1.5 Hz), 6.10 (1H, bs), 5.49 (2H, bs), 2.96 (3H, d, *J* = 4.9 Hz); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 170.0, 148.6, 132.2, 127.1, 117.3, 116.6, 116.3, 26.5; Mass spectrum (EI$^+$) m/z 150 (M); FTIR ν_{max} (cm^{-1}): 3478, 3424, 3304, 2939, 1616, 1585, 1535, 1488, 1447, 1407, 1305, 1259, 1171, 1158, 1037, 1007, 938, 859, 813, 679, 658, 561, 530, 496, 431.

2-(2,5-Dichloropyrimidin-4-ylamino)-*N*-methylbenzamide (10)

A stream of 2-amino-*N*-benzeneamide (**16**, 0.94 g, 6.25 mmol), 2,4,5 trichloropyrimidine (1.26 g, 6.88 mmol), iPr_2NEt (1.21 g, 9.38 mmol) in isopropanol (200 mL) solution was passed through a Vapourtec R2+ reaction system at 0.25 mL.min^{-1}. The instrument was fitted with two 10 mL PFA coils, maintained at 100 °C and 4 bar of pressure. HPLC analysis of the product stream, from first-pass, revealed 71% product **10** with regard to 2-amino-N-benzeneamide (**15**). The volume of the resulting product stream was reduced in half, *via* concentration under reduced pressure, to give a white slurry. Isolation of the product **10** by vacuum filtration afforded a creamy white solid (0.54 g, 62%).

^{1}H NMR (d_6-DMSO, 400 MHz): δ 12.12 (1H, s), 8.86 (1H, m), 8.51 (1H, d, *J* = 6 Hz), 8.46 (1H, bs), 7.80 (1H, dd, *J* = 7.9, 1.5 Hz), 7.61 (1H, t, *J* = 7.6 Hz), 7.23 (1H, t, *J* = 7.6 Hz), 2.82 (3H, d, *J* = 4.5 Hz); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 169.2, 157.1, 156.7, 155.8, 138.6, 132.3, 128.6, 123.8, 121.8, 121.4, 115.4, 26.8; Mass spectrum (ESI, +ve) 298 *m/z* [(M + H)$^+$, 22%], 297 (24), 157 (100); FTIR ν_{max} (cm^{-1}): (cm^{-1}) 3376, 2846, 1645, 1585, 1531, 1463, 1416, 1333, 1260, 1191, 1159, 1106, 1037, 925, 831, 794, 681, 657, 546, 526, 410.

2-((5-Chloro-2-((4-morpholinophenyl)amino)pyrimidin-4-yl)amino)-*N*-methylbenzamide (6) – CTx0-0152960

To a quartz walled Smith tube was charged with 2-(2,5-dichloropyrimidin-4-ylamino)-*N*-methylbenzamide (**10**) (476 mg, 1.60 mmol), 4-morphoaniline (**9**) (0.158 g, 0.76 mmol) and then *n*-butanol (4 mL). The ensuing slurry was crimp capped and heated in the microwave (Biotage) at 150 °C for 20 min. After cooling to 18 °C, the ensuing reaction mixture was concentrated under reduced vacuum to afford a light greenish-brown solid. Subjection of this material to flash chromatography (silica, 0:1 → 1:9 *v*/*v* methanol/dichloromethane gradient elution) and concentration of the relevant fractions (R_f = 0.4 in 1:20 *v*/*v* methanol/dichloromethane) gave the title compound (0.169 g, 51%) as an off-white coloured solid (slow decomposition from 180 °C).

^{1}H NMR (d_6-DMSO, 400 MHz): 11.59 (1H, s), 9.22 (1H, s), 9.75-9.74 (m, 2H), 8.16 (1H, s), 7.75-7.74 (3H, m), 7.13 (1H, t, J = 6 Hz), 6.89 (2H, d, *J* = 8.9 Hz), 6.89 (2H, d, *J* = 6.9 Hz), 3.75 – 3.74 (4H, m), 3.06 – 3.04 (4H, m), 2.81 (3H, d, *J* = 6 Hz); ^{13}C NMR (d_6-DMSO 151 MHz) δ 170.3, 158.5, 155.6, 153.8, 147.7, 139.2, 133.0, 131.3, 127.4, 122.3, 121.9 (2C), 121.9, 121.5, 116.7 (2C), 104.9, 50.5 (2C), 45.1 (2C), 25.4; Mass spectrum (ESI, +ve) 460 [(M+Na)$^+$, 15%], 462 (8), 438 [(M), 65], 256 (54), 178 (100); FTIR ν_{max} (cm^{-1}) 3374, 2923, 2854, 1682, 1601, 1562, 1515, 1448, 1416, 1228, 1161, 1081, 1048, 1024, 1001, 824, 759, 593, 531.

2-((5-Chloro-2-((4-(piperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)-*N*-methylbenzamide (7) – CTx-0294885

To a quartz walled Smith tube was charged with 2-(2,5-dichloropyrimidin-4-ylamino)-*N*-methylbenzamide (**10**) (0.159 g, 0.54 mmol), 4-(piperazin-1-yl)aniline (**9**) (0.103 g, 0.50 mmol), *n*-butanol (4 mL) and then HCl solution (4 drops of a 4M dioxane solution). The ensuing slurry was crimp capped and heated in the microwave (Biotage) at 150 °C for 1 h. After cooling to 18 °C, the ensuing reaction mixture was concentrated under reduced vacuum to afford a light white solid. Subjection of this material to flash chromatography (Grace Reveleris® amino column, 0:1 → 1:9 *v*/*v* methanol/dichloromethane gradient elution) and concentration of the relevant fractions (R_f = 0.4 in 1:9 *v*/*v* methanol/dichloromethane) gave the title compound as an off-white coloured solid (0.102 mg, 47%), slow decomposition from 159 °C.

^{1}H NMR (600 MHz, d_4-MeOD) δ 8.71 (1H, d, *J* = 8.3 Hz), 8.03 (s, 1H), 7.69 – 7.63 (m, 1H), 7.45 – 7.42 (3H, dd, *J* = 19.3, 8.2 Hz), 7.12 (1H, t, *J* = 7.3 Hz), 6.97 (2H, d, *J* = 8.9 Hz), 3.13 (4H, d, *J* = 5.1 Hz), 3.04 (4H, d, *J* = 4.9 Hz), 2.94 (s, 3H). ^{13}C NMR (151 MHz, d_4-MeOD) δ 170.3, 158.5, 155.6, 153.8, 147.7, 139.2, 133.0, 131.3, 127.4, 122.3, 121.9 (2C), 121.9, 121.5, 116.7 (2C), 104.9, 50.5 (2C), 45.1 (2C), 25.4; Mass

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spectrum (ESI, +ve) *m/z* 459 [(M+Na)$^+$, 19%], 437 [(M), 100], 439 (33), 304 (19), 256 (38), 219 (20), 178 (100), 156 (95); FTIR ν_{max} (cm^{-1}): 3254, 1622, 1570, 1514, 1423, 1228, 827, 771, 750.

Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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