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Original article

Synthesis and evaluation of a focused library of pyridine dicarbonitriles against prion disease

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Abstract

We report the preparation and screening of a set of 55 pyridine dicarbonitriles as potential prion disease therapeutics. Use of microwave irradiation in an attempt to improve the synthesis typically led to only small enhancement in yields but gave cleaner reactions facilitating product isolation. The library was analysed for binding to human prion protein (huPrP^{C}) by surface plasmon resonance and for inhibition of the formation of its partially protease resistant isoform PrP^{Sc} in mouse brain cells (SMB). A total of 26 compounds were found to bind to huPrP^{C} whilst 12 showed discernable inhibition of PrP^{Sc} formation, five displaying EC_{50}s in the range 2.5–9 μM. Two compounds were found to reduce PrP^{Sc} levels to below 30% relative to an untreated control at 50 nM.

Keywords: Prion protein; TSE; vCJD; Pyridine dicarbonitriles; Microwave irradiation; SPR

1. Introduction

Prion diseases are a family of invariably fatal neurodegenerative disorders affecting humans and animals including, cattle, sheep, cervids (deer and elk) and mink [1]. These maladies, known as transmissible spongiform encephalopathies (TSEs), pose a significant risk to public health due to transmission both to animals and humans. As such, they have been the focus of much interest in recent years following discovery of a new variant of the human TSE Creutzfeldt–Jacob disease (vCJD), thought to have been triggered by the consumption of contaminated beef products [2–4]. Iatrogenic transmission has also been reported via contact with contaminated neurosurgical instruments, tissue grafts and blood products [5]. TSEs are associated with a post-translational conversion of the cell-surface glycosylphosphatidylinositol (GPI)-anchored protein PrP^{C} to a partially protease resistant isoform denoted PrP^{Sc}. No effective therapy currently exists for the prevention of either infection or disease progression [6]. As a part of an ongoing medicinal chemistry research programme we are seeking to identify small drug-like molecules that bind to PrP^{C} and stabilise it against this conversion, with the aim of identifying novel prion disease therapeutics.

In 2000 Perrier et al. reported a number of compounds exhibiting inhibition of PrP^{Sc} formation in mouse neuroblastoma cells (Fig. 1) [7]. Using surface plasmon resonance (SPR) we identified the pyridine dicarbonitrile substructure common to these actives as a source of PrP^{C} binders, and recently reported the synthesis and SPR screening of a small library of such pyridine dicarbonitriles [8]. Here, we report on our attempts to further optimise the synthesis, and on the construction of a second more focused library followed by quantitative biological analysis.

2. Synthesis

Reported yields for the synthesis of pyridine dicarbonitriles, both by us and others, are at best moderate [8,9]. We

Abbreviations: PrP^{C}, normal cellular prion protein or PrP-sen; PrP^{Sc}, disease-causing isoform or PrP-res; SMB, Scrapie-infected mouse brain; SPR, surface plasmon resonance; vCJD, variant Creutzfeldt–Jakob disease.

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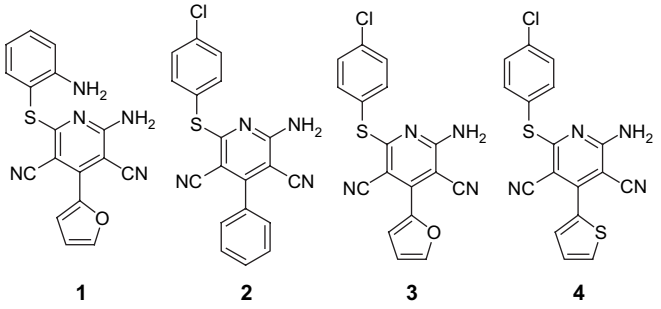


Fig. 1. Dicarbonitriles reported as active in mouse ScN2a cells.

therefore sought to improve the chemistry so as to facilitate further library synthesis. Since the first pioneering publications in 1986 [10,11], there have been numerous reports of microwave irradiation (MWI) being used in place of conventional heating, often resulting in vastly reduced reaction times and less side reactions [12]. One of the advantages of synthesis under MWI conditions is that solvent is not required (but can be used if desired). It was thus decided to investigate the effect of MWI upon the one-pot synthesis of pyridine dicarbonitriles to see what advantages might be gained from the use of this technique.

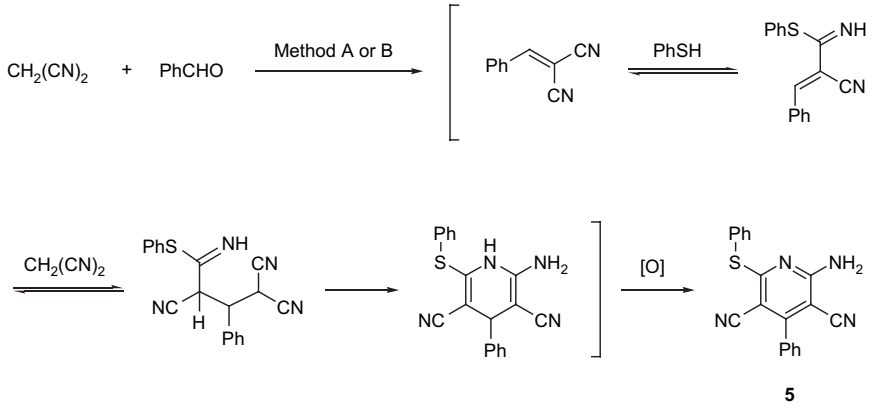
The one-pot synthesis we described previously [8] involves refluxing a solution of aldehyde, thiol and malononitrile (in a ratio of 1:1:2) with 10 mol% piperidine in ethanol for 24 h, followed by stirring in air for a further 3 h to allow oxidation of the 1,4-dihydropyridine so formed to the final product. In our hands the one-pot synthesis of 2-amino-4-phenyl-6-phenylsulfanylpyridine-3,5-dicarbonitrile **5**—derived from benzaldehyde, thiophenol and malononitrile — resulted in a 43% isolated yield [8]. To develop the microwave methodology, initial investigations were carried out on the solvent-free reaction of these same reagents under MWI (Scheme 1).

Using Method B, irradiation at 90 °C for 10 min led to product **5** in 31% yield, whereas extending the irradiation period to 1 h resulted in a higher yield of 51%. Longer reaction time (2 h) offered no further advantage, resulting in only a 38% yield. Irradiation at higher temperatures (150 and 200 °C) for 15 min also proved inferior, giving just 38% yield of product in both cases. Under the best conditions (solvent-free, MWI at 90 °C) compound **6**, derived from 3-chlorothiophenol, was isolated in a slightly improved 29% yield (compared to 23% for Method A), and in 32% yield when ethanol was used as solvent.

The solvent-free conditions described above (Method B; MWI for 1 h, followed by aerobic oxidation for 3 h) were then applied to the synthesis of some of the compounds of interest from our previous study which had hitherto been produced in poor yield, or not at all. It was pleasing to note that in the cases of compounds **6**–**9**, yields were seen to rise by an average of 15%; however, MWI conditions offered no advantage for compounds **10** and **11**. Di-hydroxyphenyl compound **8** had proven inaccessible under classical conditions (Method A), so it was gratifying to note that it could now be isolated successfully. Furaldehyde derivative **12** remained unobtainable, however (Table 1).

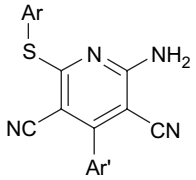
As lead structures **1** and **3** — containing a furyl substituent at the 4-position — showed cell line activity, further exploration of the synthesis of 4-(2-furyl) substituted dicarbonitriles was considered important. Abdel-Latif et al. reported the reaction of Knoevenagel adduct **13** with malononitrile, thiol **14** and piperidine was refluxed with ethanol for 4 h to form the desired 4-furyl compound **1** in 67% isolated yield (Scheme 2) [13]. However, this result could not be reproduced when the same experiment was attempted in our laboratories. We found that when **13** was refluxed in ethanol with malononitrile, various thiols and piperidine, only a complex mixture of compounds was observed from which none of the desired products could be isolated. The procedure was repeated at room temperature and after several hours a red crystalline side product **15** was observed, isolated in 36% yield and confirmed by TLC as a major component of the previously obtained mixtures.

This result implied preferential addition of further malononitrile, rather than the thiol, to intermediate **13** and therefore led us to investigate the order of reagent addition, aimed at reducing the amount of free malononitrile present during the reactions. The best conditions were found to be heating a two-fold excess of thiol with malononitrile and catalytic amounts of piperidine for 30 min at reflux before addition of



Scheme 1. Synthesis of pyridine dicarbonitriles. Method A: malononitrile, piperidine, ethanol were refluxed for 24 h, then underwent aerobic oxidation for 3 h. Method B: malononitrile, piperidine, MWI for 10–120 min at 90 °C, then underwent aerobic oxidation for 3 h.

Table 1
Comparison between yields obtained by classical and MWI methods



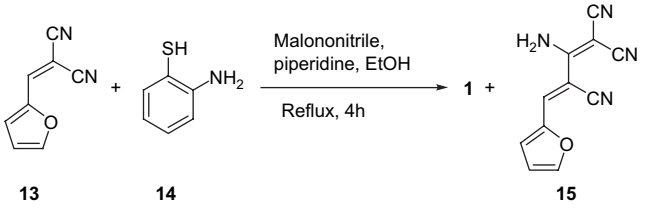
Compound No.	Ar	Ar′	Yield[a] [%]	Yield[b] [%]
5	Phenyl	Phenyl	43	51
6	3-Cl-ph	Thiophene	23	29
7	3-Cl-ph	4-Cl-ph	25	41
8	Benzyl	3,4-OH-ph	0	23[c]
9	4-OH-ph	Thiophene	27	43
10	4-OH-ph	3-OH-ph	20	21
11	Benzyl	Thiophene	19	21
12	Phenyl	Furyl	0	0

[a] Yield from Method A.
[b] Yield from Method B (MWI mediated).
[c] Piperidine (2.1 equiv).

adduct **13**, maintaining reflux. After a further 30 min the reaction mixture was allowed to cool to room temperature and undergo aerobic oxidation after which the products were obtained as crystalline solids by careful addition of water, albeit in low yields. Though the remaining mother liquors still contained product, second crops of solids which were collected showed high degrees of impurities and as such were discarded. Nonetheless, using the methodologies outlined here, a selection of previously elusive pyridine dicarbonitriles derived from furaldehyde was constructed and added to the library for bioassay.

3. Library synthesis

In our previous studies we commented on an interesting relationship in which relocating the substituent on the phenylthiol group from the *meta*- to the *para*-position resulted in a loss of binding. One of the foci of this second library was to further investigate this trend, and as such, pairs of *meta*- and *para*-substituted thiophenols were employed. As literature compounds **1** and **3** possessed a furyl group in the 4-position it was also appropriate, having overcome the synthetic barrier to these compounds encountered previously, to enrich the library with some relevant analogues. Due to the reported cell line activity of compound **11** at 24 μM [8], benzyl mercaptan and thiophene-2-carboxaldehyde were utilised. Similarly, thiophenols featuring a methyl ester moiety in the *meta*- or *para*-position were considered necessary, along with 4-formylbenzoic acid methyl ester, as preliminary screening had revealed potency in the products derived from these materials.



Scheme 2. Synthesis of 4-furyl substituted pyridine dicarbonitriles.

Microwave reactions were carried out using a Personal Chemistry SmithCreator™ system. Achieving the minimum reaction volume required by our microwave device (2.0 mL) required relatively large quantities of reagents under solvent-free conditions (a total of six compounds were prepared in this way). For this reason, 36 of the library members were synthesised in ethanol (16 compounds at 1 mmol, one at 2 mmol and 19 at 3 mmol in 2.0 mL). Another five compounds were synthesised with conventional heating, and those 4-(2-furyl) compounds not available by these methods were synthesised by the alternative procedure described above (six compounds). In total, a library of 45 pyridine dicarbonitriles was prepared. As syntheses using poly-hydroxybenzaldehydes had proven unproductive, 1,4-benzodioxan-6-carboxaldehyde and piperonal were introduced as oxygen-bearing alternatives, giving products in reasonable yields in the majority of cases. However, in the case of the reaction between piperonal and 2-hydroxythiophenol the desired product could not be isolated.

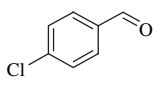
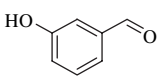
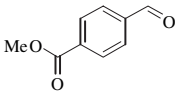
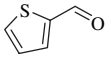
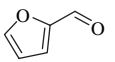
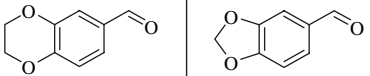
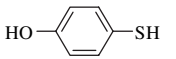
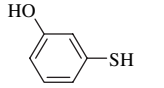
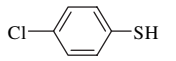
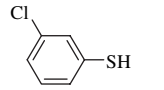
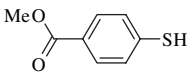
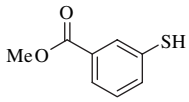
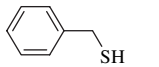
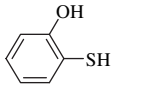
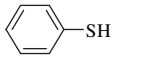
4. Screening (SPR) methodology

Surface plasmon resonance (SPR) was carried out using a BIAcore 3000 (BIAcore, Uppsala, Sweden) equipped with a CM5 sensor chip (carboxymethylated dextran). The methodology was as reported previously [8,14]. Interactions were measured with two forms of prion protein, full length human ($huPrP^C$) and full length murine ($moPrP^C$). Compounds were screened at 40 μM in running buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20) containing 6.5% DMSO. DMSO calibration using buffer samples containing 5.5–7.5% DMSO was carried out to correct solvent effects. Compounds producing a response of more than 2.5 response units (RU) were considered to be binders. Binding affinities are expressed as $\%RU_{max}$, that is, as a percentage of the theoretical maximum response for 1:1 protein–ligand binding. No data is presented for compounds either seen to interfere with the chip surface, or for those that could not be washed off the protein (Table 2).

5. Screening (SMB cells) methodology

Compounds were screened for inhibition of PrP^{Sc} formation in SMB cells of mesodermal origin, the procedure used being based upon that reported by Rudyk et al. [15]. A persistently infected mouse cell line (SMB), cloned originally from scrapie-infected mouse brain but of non-neuronal origin [16], was used. Cells were grown in tissue culture treated plastic dishes in Medium 199 (phenol red free), supplemented with 10% newborn calf serum (heat inactivated), 5% foetal calf serum (heat inactivated) and penicillin–streptomycin at 10 mg L^{-1} at 37 °C in an atmosphere of 5% CO_2 in air at 95% relative humidity. Medium was changed every 3rd or 4th day, and every 7 days confluent cells were passaged using

Table 2
Screening data from a library of pyridine dicarbonitriles

Compound No. (Yield [%]) [%RU_{max} 40 μM; huPrP^{C}, moPrP^{C}] PrP^{Sc} [%] (conc. [μM])								
		A	B	C	D	E	F	G
	1	**29** (29[c]) [–][a] 90 ± 8 (10)	**10** (21[c]) [34.0, 43.1] 85 ± 8 (10)	**16** (42[e]) [Non-Binder] 45 ± 15 (7.5)	**9** (43[b]) [Non-Binder] 86 ± 22 (10)	**48** (7[f]) [21.9, 19.6] 24 ± 6 (0.05)	**54** (35[d]) [Non-binder] Toxic	**40** (31[d]) [–][a] 82 ± 3 (10)
	2	**30** (28[c]) [–][a] 73 ± 0 (10)	**51** (38[c]) [Non-Binder] 36 ± 10 (5); Toxic (10)	**17** (27[c]) [8.4, 0.0][h] 77 ± 5 (10)	**55** (28[d]) [–][a] 77 ± 15 (10)	Not isolated	**56** (46[d]) [–][a] 86 ± 10 (10)	**41** (34[d]) [250.0, 137.5] 75 ± 11 (10)
	3	**31** (37[c]) [19.3, 1.6] 72 ± 7 (10)	**35** (13[c]) [15.7, 8.9] EC_{50} ~ 3 μM	**18** (52[c]) [10.6, 2.4] 73 ± 4 (10)	**4** (23[b]) [8.8, 0.0][h] EC_{50} ~ 3 μM	**3** (14[f]) [37.0, 44.8] 73 ± 3 (10)	**38** (40[d]) [Non-binder] 68 ± 18 (10)	**39** (46[d]) [–][a] 67 ± 5 (10)
	4	**7** (41[c]) [118.7, 52.3] 71 ± 13 (10)	**57** (37[c]) [–][a] 36 ± 4 (5); Toxic (10)	**19** (46[e]) [Non-binder] 46 ±8 (5)	**6** (29[b]) [123.6, 72.2] 84 ± 13 (10)	**58** (9[f]) [–][a] 94 ± 0 (10)	**52** (34[d]) [11.3, 4.3] EC_{50} ~ 2.5 μM	**42** (39[d]) [78.6, 10.5] 96 ± 10 (10)
	5	**24** (43[c]) [Non-binder] Toxic	**25** (41[c]) [Non-binder] 70 ± 8 (10)	**20** (14[e]) [Non-binder] EC_{50} ~ 9 μM	**26** (17[b]) [Non-binder] 90 ± 12 (10)	**27** (6[f]) [Non-binder][a] 69 ± 12 (10)	**28** (44[d]) [6.0, 0.0] [h] 23 ± 6 (0.05)	Not isolated
	6	**32** (41[c]) [6.2, 0.0][h] 63 ± 25 (1)	**59** (41[c]) [–][a] 123 ± 18 (10)	**21** (52[e]) [Non-binder] EC_{50} ~ 8 μM	**60** (46[b]) [–][a] 87 ± 3 (10)	**49** (16[f]) [26.9, 2.2] 69 ± 1 (10)	**53** (43[d]) [–][a] 44 ± 8 (0.05)	**43** (50[d]) [155.9, 70.4] 75 ± 20 (10)
	7	**33** (40[c]) [8.6, 0.0][h] 92 ± 0 (10)	**61** (25[e]) [96.5, 112.2] 113 ± 17 (10)	**22** (39[c]) [9.3, 0.0][h] 107 ± 2 (10)	**11** (21[b]) [55.5, 52.6] 81 ± 16 (10)	**50** (6[f]) [–][a] 111 ± 10 (10)	**62** (40[d]) [–][a] 94 ± 15 (10)	**44** (46[d]) [–][a] 79 ± 1 (10)
	8	**34** (43[d]) [9.1, 0.0][h] 76 ± 14 (10)	Not isolated	**23** (42[d]) [5.7, 0.0][h] 72 ± 0 (10)	**46** (30[d]) [16.9, 6.1] 65 ± 9 (10)	Not isolated	**47** (20[d,g]) [13.6, 4.9] 89 ± 9 (10)	Not isolated
	9						**63** (29[d]) [27.9, 0.0][h] 61 ± 17 (10)	**45** (39[d]) [Non-binder] 86 ± 3 (10)

[a]Compound interfered with the chip surface or could not be washed off the protein. [b]Solvent free conditions. [c]1 mmol in 2.0 mL EtOH. [d]3 mmol in 2.0 mL EtOH. [e]Conventional heating.[8] [f]Alternative method.
[g]Crude yield, <80% pure by HPLC (see experimental section). [h]Binding observed to huPrP^{C} only and not moPrP^{C}.

0.05% trypsin and 0.002% EDTA at a split ratio of 4. To assess the effects of compounds cells were distributed into 96-well cluster plates at 3×10^4 cells per well and incubated for 24 h to allow cell attachment. The compounds were diluted to 400 times the required concentration in DMSO as stock solutions then transferred, at a 20-fold dilution, into Hank's balanced salt solution. This solution was then transferred at a further 20-fold dilution into the cell medium. The cells were incubated with the compound-containing medium for 5 days.

After 5 days cell viability was assessed by the MTT assay following the standard protocol supplied with the reagent (Sigma). For dot blot analyses cells were extracted using lysis buffer (10 mM Tris–HCl [pH 7.6], 100 mM NaCl, 10 mM EDTA, 0.5% v/v NP40 and 0.5% w/v sodium deoxycholate), and the content of the well loaded onto a nitrocellulose membrane (0.45 μm) under gentle vacuum at a total cellular protein concentration of approximately 30–40 μg/well (determined by the Bradford assay following the protocol supplied with the reagent – Sigma). The membrane was air dried and subjected to 75 $\mu g\,mL^{-1}$ proteinase K digestion for 1 h at 37 °C. The reaction was stopped with 1 mM phenylmethylsulfonyl fluoride (PMSF) in 20 mM Tris-HCl-buffered saline (TBS), the membrane washed extensively with TBS, and immersed in 1.8 M guanidine thiocyanate in TBS for 10 min at room temperature. After further washing with TBS the membrane was blocked using 5% fat-free milk powder in phosphate buffered saline (PBS), processed with 0.2 $\mu g\,mL^{-1}$ mouse monoclonal anti-PrP 6H4 (Prionics) and developed using an ECL kit (Amersham Pharmacia Biotech).

Each experiment was carried out in triplicate and an average value for PrP^{Sc} concentration calculated, relative to an untreated control, together with a standard deviation. Compounds were initially screened at 1 and 10 μM and were considered to be active if PrP^{Sc} levels were reduced to less than 70% of that of the untreated control after 5 days' exposure. The amounts of PrP^{Sc} remaining as a percentage relative to the control are given in Table 2. Determination of EC_{50} values was carried out by assay over a range of concentrations and calculated where an acceptable dose–response curve was observed. If such data was inconclusive, the lowest active concentration supplanted the initial screening result in Table 2.

6. Results and discussion

Of the 53 compounds shown in Table 2, 26 were binders to PrP^C, 13 were non-binders and 14 interacted with the chip surface or could not be removed from the immobilised protein using standard regeneration buffer. Binding data is reported in Table 2. As mentioned above, the purported detrimental effect of *para*- vs. *meta*-substituted thiophenols on binding was one of the points of investigation for the present library; however, it can be seen that such a pattern was not observed amongst the data reported here.

The strongest binders to PrP^C were found amongst those compounds derived from the aldehyde piperonal (column G, Table 2), or the thiols 3-chlorothiophenol (row 4) and benzyl mercaptan (row 7), though no clear relationship between structure and binding affinity is apparent. Indeed, considering the library as a whole, no obvious such correlation is evident, though in some cases direct comparison between different structural features was hampered by unavailability of data through synthetic inaccessibility or interaction of compounds with the chip surface (e.g. comparison of a thiophene or furan substituent at the 4-position). Nonetheless, given the lack of any recognisable trends in the binding results it could be postulated that the data obtained are indicative of non-specific binding to the proteins.

To investigate this further, simple kinetic experiments were carried out by measuring binding to $huPrP^C$ ($\%RU_{max}$) as a function of compound concentration. When applied to the strongest binders shown in Table 2, however, such measurements were either inconclusive or indicative of non-specific binding (data not shown); such results are not conclusive, however. A number of our other screening subjects have been assessed for binding to PrP^C using a fluorescence-based assay [17], taking advantage of the natural fluorophore present in the protein. This work has proven that some compounds displaying non-specific binding to PrP^C under the SPR conditions do, in actual fact, show specific (i.e. 1:1) binding to the protein when assessed using the alternative fluorescence assay. Thus, although the SPR assay is useful as an initial screening tool, evaluation of protein–small molecule interactions appears to approach the detection limit of the Biacore instrument making reliable determination of K_d values difficult.

Cell line screening was carried out using SMB cells, a persistently infected murine cell line acting as a host cell for PrP^{Sc} [15,16]. In this cell line, active replication of the PrP^{Sc} infectious agent occurs leading to its accumulation in readily detectable amounts. Such host cell lines act, to a degree, as a valuable alternative to animal models of scrapie thereby constituting a useful initial screening tool in the search for novel prion disease therapeutics. Cell line screening results are shown together with the binding data in Table 2 (active compounds highlighted). It was pleasing to note the compound **4** (entry D3 in Table 2), reported previously [7] as an anti-prion agent by Perrier et al., also showed efficacy in our cell line assay. Though an EC_{50} of 3 μM was observed (Fig. 2), this compound did demonstrate some toxicity towards the cells at concentrations not far above the EC_{50} value (dotted line denotes cell viability). Two related compounds (**35**; entry B3, and **52**; entry F4) for which a similar EC_{50} value could be deduced are also derived from chlorophenyl substituents at the 4-position (Fig. 2), but did not suffer from the problems with toxicity observed for **4**. Surprisingly, related structure **3** (entry E3), bearing a furan rather than a thiophene substituent at the 4-position, was found to be inactive under our assay conditions.

In addition to **35**, two other compounds derived from 3-hydroxybenzaldehyde, **51** (entry B2) and **57** (entry D2), showed clear evidence of activity at 5 μM and below but became toxic to the cells as the concentration approached 10 μM. Nonetheless, the 3-hydroxyphenyl substituent at the 4-position may serve as a useful lead for further SAR studies if analogues with lower toxicity can be developed whilst retaining cell line activity.

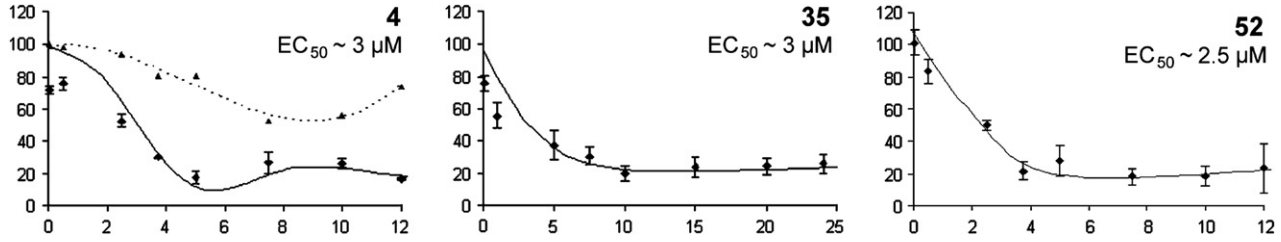


Fig. 2. Dose–response curves for compounds **4**, **35** and **52**, *x*-axis values correspond to compound concentration [μM], *y*-axis values denote percentage of PrP^{Sc} remaining relative to an untreated control. For compound **4**, dotted line represents cell viability.

Similarly, three compounds derived from 2,3-dihydrobenzo-[1,4]dioxine-6-carbaldehyde (column F, Table 2) showed good cell line activity, thereby identifying this substituent at the 4-position as an additional useful lead. Despite its similar structure, none of the compounds derived from piperonal (column G) proved active, perhaps as a result of the proneness of the benzo[1,3]dioxolane group towards facile hydrolysis or metabolisation. In addition to **52** (entry F4, $EC_{50} \sim 2.5$ μM), derived from the 2,3-dihydrobenzo[1,4]dioxine-6-carbaldehyde, compounds **28** (entry F5) and **53** (entry F6) significantly reduced PrP^{Sc} levels at 50 nM. Interestingly, though **28** eliminated most of the PrP^{Sc} at lower concentrations, PrP^{Sc} levels recovered at higher concentrations of the drug (Fig. 3); **53** gave a comparable dose–response curve, though reduction of PrP^{Sc} levels was not as pronounced at lower concentrations. The dose–response properties shown by these compounds are not understood, but since both contain a methyl ester substituent on the thiophenyl group at the 6-position, it may be hypothesised that metabolism through esterase activity results in loss of efficacy as drug concentration increases.

A similar trend is seen with compound **48** (entry E1), whose dose–response curve is also shown in Fig. 3. In this case PrP^{Sc} levels do not recover as quickly as concentration increases – still only reaching about 50% relative to the control at 12 μM – perhaps indicating some slower metabolism of the compound than for the examples described above.

Four other compounds containing methyl ester groups also showed some efficacy in the cell line assay. Compound **21** (entry C6), containing a methyl ester moiety on the substituents at both the 4- and 6-positions, showed an apparent EC_{50} of 8 μM but gave a biphasic dose–response curve (Fig. 3). Known as hormesis [18], this phenomenon is not fully understood, but is quite likely due to hydrolysis of the ester groups in the present case. Compound **20** (entry C5) – also containing a methyl ester group – gave a similar dose–response curve with an EC_{50} of 9 μM; and relatedly, **19** (entry C4) and **16** (entry C1) displayed evidence for similar activity though a clear EC_{50} value could not be deduced in these cases.

In particular, incorporation of a methyl ester group onto the aromatic ring at the 4-position (column C, Table 2) gave rise to a striking number of active compounds. As a whole, structures where a methyl ester group was introduced (at the *para*-position of either the thiol or aldehyde, or the *meta*-position of the thiol) showed some promising initial results but their evaluation was hindered by apparent metabolisation of the compounds. Given the activity observed though, structurally similar analogues which are not as readily metabolised would constitute an interesting part of any further SAR studies.

Though several cell line active compounds are reported here, no obvious correlation exists between PrP^{Sc} binding affinity and such activity. Although, as mentioned above, it cannot yet be confirmed whether any specific binders to PrP^{Sc} are present among the library under consideration, our observations do infer that more than one mode of action is present within the set of active compounds. Despite the considerable challenge, further studies to ascertain the different modes of action through which these compounds are acting would thus prove most valuable.

7. Conclusions

It has been postulated [7] that a suitable ligand may stabilise PrP^{C} against conversion to PrP^{Sc}. Within our library of 2-aminopyridine-3,5-dicarbonitriles, no obvious such correlation between binding and cell line activity was evident, though some of the active compounds reported were found to be

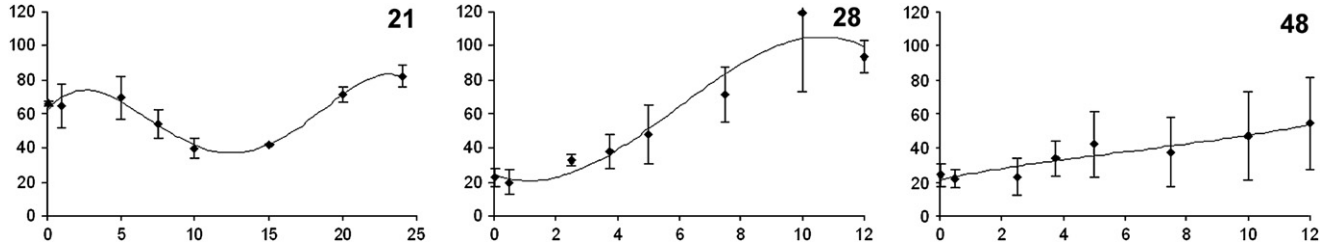


Fig. 3. Unusual dose–response profiles exhibited by compounds **21**, **28** and **48**, *x*-axis values correspond to compound concentration [μM], *y*-axis values denote percentage of PrP^{Sc} remaining relative to an untreated control.

weak to moderate binders to PrP^C, including two **35** and **52** with an EC_{50} in the range of 3 μM.

Modification of the 4- and 6-positions did not lead to an established SAR at the present stage, but did generate some interesting leads which will provide information for directing further studies. Analysis of the distribution of actives in Table 2 indicates that the substituent at the 4-position of the pyridine dicarbonitriles may be more influential upon activity than that at the 6-position. Five of the active compounds contained a *meta*- or *para*-chlorothiophenyl group at the 6-position, making these substituents of interest for further investigation. Replacement of the chlorine atom with a methyl ester group in these compounds retained some of, or even improved upon, their activity, but such potency appeared to be lost through metabolism as drug concentration increased thereby necessitating the development of more robust structural analogues. Favoured substituents at the 4-position were 6-(2,3-dihydrobenzo[1,4]dioxinyl), 3-hydroxyphenyl, and – especially – *para*-(carboxymethyl)phenyl. Should the problems of toxicity and metabolism, respectively, of compounds containing the latter two groups be overcome by the preparation of suitable analogues, genuine drug candidates for the treatment of prion disease may emerge.

We have herein documented two compounds (**35** and **52**) of equivalent activity but lower toxicity when compared to the previously reported [7] active compound **4**, thereby achieving improvement upon this earlier anti-prion compound. Within our library, a number of structural features have been identified to assist in the development of more potent compounds, and three compounds (**28**, **48** and **53**) have been found to effect significant clearance of PrP^{Sc} from infected cells in the nanomolar concentration range.

8. Experimental

8.1. Chemistry

8.1.1. General procedures

Melting points were measured using a Bibby-Sterilin SMP10 melting point apparatus. Accurate mass and nominal mass measurements were measured using a Waters-Micromass LCT electrospray mass spectrometer. TLC was performed using aluminium backed silica gel 60 plates (0.20 mm layer). Flash column chromatography was carried out using Fluorochem silica gel 60 Å. All compounds were isolated in >95% purity unless otherwise stated. (As determined by HPLC under two sets of conditions – HPLC 1: Luna 5 μ C18 150 × 4.6 mm, 5–95% acetonitrile in water [0.1% TFA] over 4 min, 1 mL min^{-1}, 20 μL injection. Detection was at 256 nm, run time 10 min. HPLC 2: Altima HP 3 μ C18 EPS 150 × 4.6 mm, 35–98% acetonitrile in water [0.1% TFA] over 4 min, 1.0 mL min^{-1}, 20 μL injection. Detection was at 256 nm, run time 11 min.) All reagents were purchased directly from commercial sources and used as supplied. Compound **13** was prepared according to the literature [18], Mpt., 72–73 °C (Literature [19], 72.5–73 °C). Compounds **5**, **16**, **19**, **20**, **21**, **26** and **61** were reported previously [8].

8.1.2. Microwave synthesis

SmithCreator™ Optimiser EXP reactor (Personal Chemistry, Inc.) was used. The machine consists of a continuous focused microwave power delivery system. Reaction times and temperatures are operator selectable. Sample temperature is constantly monitored by IR, pressure by a transducer on the top of the vial's septum, and the microwave power is automatically adjusted to maintain programmed temperature profiles. For the experiments reported here, ''fix hold time'' was set to ''on'' and ''absorption level'' set to ''normal''. Reactions were carried out in Smith Process Vials™ (2.0–5.0 mL).

8.2. General procedure for solvent-free MWI synthesis

To a mixture of thiol (7.00 mmol), aldehyde (7.00 mmol) and malononitrile (0.88 mL, 14 mmol) was added piperidine (0.07 mL, 0.70 mmol). The resultant mixture was heated by microwave irradiation at 90 °C for 60 min, after which the reaction mixture was dissolved in 15.0 mL ethanol. The mixture was then exposed to air and stirred at room temperature for 3 h. The precipitate formed was collected by suction filtration, washed with *n*-hexane/ethanol (1:1), and then dried under high vacuum.

8.2.1. 2-Amino-6-(3-chlorophenylsulfanyl)-4-thiophen-2-yl pyridine-3,5-dicarbonitrile (*6*)

Yield 29%, yellow powder. Mpt., 197–198 °C, decompose; ν_{max} (solid)/cm^{-1}, 3372.0, 3091.8, 2360.1, 2203.9, 1611.3, 1574.9, 1542.5, 1506.0, 1458.4, 1429.7, 1408.9, 1314.0, 1256.3, 1239.4, 1123.8, 1080.3, 1064.0, 994.0, 911.6, 866.4, 850.5; δ_H/ppm (250 MHz, d_6-DMSO), 7.25–7.35 (1H, m, Ar-H), 7.45–7.65 (4H, m, Ar-H), 7.69 (1H, t, $J = 2.0$, Ar-H), 7.92 (2H, bs, NH_2), 7.97 (1H, d, $J = 3.0$); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.5, 93.7, 115.6, 115.9, 128.4, 129.8, 130.3, 131.5, 131.8, 133.1, 134.0, 134.1, 134.6, 151.4, 160.4, 166.6; *m/z* (EI), 368 (M^+); found 367.996141 ($C_{17}H_9ClN_4S_2$ M^+, requires 367.995718).

8.2.2. 2-Amino-6-(4-hydroxyphenylsulfanyl)-4-thioph-en-2-yl pyridine-3,5-dicaronitrile (*9*)

Yield 43%, yellow crystalline solid. Mpt., 225–226 °C decompose; ν_{max} (solid)/cm^{-1}, 3468.7, 3324.3, 3216.1, 3159.3, 3094.2, 2361.9, 2338.2, 2209.4, 1624.9, 1583.4, 1545.4, 1508.4, 1498.0, 1433.9, 1401.8, 1357.8, 1275.4, 1259.7, 1228.3, 1170.0, 1089.0, 1045.6, 1005.7, 876.1, 831.2, 812.5; δ_H/ppm (250 MHz, d_6-DMSO), 6.87 (2H, d, $J = 8.0$, Ar-H), 7.29 (1H, t, $J = 4.0$, Ar-H), 7.37 (2H, d, $J = 9.0$, Ar-H), 7.57 (1H, d, $J = 4.0$), 7.79 (2H, bs, NH_2), 7.95 (1H, d, $J = 5.0$, Ar-H), 9.99 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 86.4, 92.6, 114.8, 115.2, 115.4, 116.6, 127.9, 130.7, 131.2, 132.8, 137.1, 150.7, 159.1, 159.8, 168.2; *m/z* (EI), 350 (M^+); found 350.029786 ($C_{17}H_{10}N_4OS_2$ M^+, requires 350.029605).

8.2.3. 2-Amino-6-benzylsulfanyl-4-thiophen-2-yl-3,5-dicarbonitrile (*11*)

Yield 21%, yellow powder. Mpt., 204–205 °C; ν_{max} (solid)/cm^{-1}, 3435.9, 3320.4, 3212.6, 2200.8, 1994.1, 1623.9, 1537.7,

1504.9, 1432.5, 1401.8, 1356.5, 1256.1, 1236.6, 1203.2, 1070.2, 1008.6, 852.6, 833.0; δ_H/ppm (250 MHz, d_6-DMSO), 4.50 (2H, s, CH_2), 7.15–7.40 (4H, m, Ar-H), 7.45–7.60 (3H, m, Ar-H), 7.94 (1H, d, $J = 2.5$, Ar-H), 8.16 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 32.3, 85.7, 93.0, 115.3, 127.3, 127.9, 128.4, 129.4, 130.8, 131.2, 132.7, 137.5, 150.7, 159.7, 166.7; *m/z* (EI), 348 (M^+); found 348.050581 ($C_{18}H_{12}N_4S_2$ M^+, requires 348.050340).

*8.2.4. 2-Amino-6-(4-chlorophenylsulfanyl)-4-thiophen-2-yl pyridine-3,5-dicarbonitrile (**4**)*

Yield 23%, yellow powder. Mpt., 226–227 °C, decompose; ν_{max} (solid)/cm^{-1}, 3475.1, 3329.9, 3219.9, 2208.6, 1635.8, 1544.8, 1525.6, 1508.5, 1472.2, 1431.8, 1403.9, 1386.3, 1355.5, 1255.2, 1235.9, 1175.8, 1087.9, 1007.7, 906.2, 835.9, 820.0; δ_H/ppm (250 MHz, d_6-DMSO), 7.30 (1H, t, $J = 4.0$, Ar-H), 7.50–7.70 (5H, m, Ar-H), 7.90 (2H, bs, NH_2), 7.97 (1H, d, $J = 5.0$, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 86.9, 93.0, 115.1, 115.4, 126.0, 127.9, 129.5, 130.9, 131.3, 132.7, 134.8, 136.7, 150.9, 159.8, 166.3; *m/z* (EI), 368 (M^+); found 367.994946 ($C_{17}H_9ClN_4S_2$ M^+, requires 367.995718); Purity: HPLC 1, 98%; HPLC 2, 90%.

*8.2.5. 3-(6-Amino-3,5-dicyno-4-thiophen-2-yl pyridin-2-ylsulfanyl)-benzoic acid methyl ester (**60**)*

Yield 46%, yellow powder. Mpt., 262–263 °C, decompose; ν_{max} (solid)/cm^{-1}, 3454.1, 3348.7, 3227.8, 2211.0, 1980.0, 1969.4, 1933.1, 1792.7, 1636.8, 1548.0, 1517.5, 1460.0, 1429.9, 1404.0, 1359.9, 1293.4, 1271.6, 1256.3, 1239.4, 1193.0, 1126.2, 1093.6, 1072.1, 1019.8, 997.7, 957.2, 886.2, 852.1, 811.9; δ_H/ppm (250 MHz, d_6-DMSO), 3.88 (3H, s, CH_3), 7.30 (1H, t, $J = 4.5$, Ar-H), 7.55–7.75 (2H, m, Ar-H), 7.75–7.95 (3H, m, Ar-H, NH_2), 7.97 (1H, d, $J = 5.5$, Ar-H), 8.05–8.15 (2H, m, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.4, 87.0, 115.1, 115.4, 127.9, 128.0, 130.0, 130.4, 131.4, 132.7, 135.6, 139.7, 151.0, 159.8, 165.5, 166.2; *m/z* (EI), 392 (M^+); found 392.039440 ($C_{19}H_{12}N_4O_2S_2$ M^+, requires 392.040169).

8.3. General procedure for MWI synthesis in ethanol (1 mmol)

To a stirred solution of aldehyde (1.00 mmol) in ethanol (2.0 mL) containing thiol (1.00 mmol) and malononitrile (0.13 mL, 2.00 mmol) piperidine (0.01 mL, 0.10 mmol) was added. The resultant solution was heated by microwave irradiation at 90 °C for 60 min, after which the reaction mixture was exposed to air, and stirred for 3 h at room temperature. The precipitate formed was collected by suction filtration, washed by *n*-hexane/ethanol (1:1), and then dried under high vacuum.

*8.3.1. 2-Amino-4-(4-chlorophenyl)-6-(3-chlorophenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**7**)*

Yield 41%, yellow powder. Mpt., 216–217 °C, decompose; ν_{max} (solid)/cm^{-1}, 3390.9, 2358.2, 2219.6, 2209.8, 1982.1, 1964.8, 1937.7, 1608.6, 1572.4, 1543.1, 1519.4, 1493.9, 1461.9, 1422.3, 1407.9, 1318.5, 1256.7, 1153.7, 1116.0, 1096.1, 1013.9, 994.8, 954.3, 859.8, 830.1, 804.3; δ_H/ppm (250 MHz, d_6-DMSO), 7.45–7.75 (8H, m, Ar-H), 7.95 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 87.4, 93.4, 114.8, 115.1, 128.9, 129.3, 129.8, 131.0, 132.7, 133.5, 133.6, 134.0, 135.4, 157.5, 159.6, 165.4; *m/z* (EI), 396 (M^+); found 396.000846 ($C_{19}H_{10}Cl_2N_4S$ M^+, requires 396.000324).

*8.3.2. 2-Amino-4-(3-hydroxyphenyl)-6-(4-hydroxyphenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**10**)*

After 20 h stirring at room temperature the product was isolated as a yellow powder (21% yield). Mpt., 224–226 °C; ν_{max} (solid)/cm^{-1}, 3399.8, 3325.5, 3223.0, 2220.1, 1639.9, 1587.9, 1542.3, 1521.3, 1496.0, 1465.6, 1442.2, 1381.3, 1340.5, 1316.1, 1269.5, 1213.3, 1169.8, 1088.0, 1039.7, 1010.7, 997.7, 948.5, 903.5, 871.1, 836.0, 817.1; δ_H/ppm (250 MHz, d_6-DMSO), 6.75–7.05 (5H, m, Ar-H), 7.25–7.50 (3H, m, Ar-H), 7.73 (2H, bs, NH_2), 9.89 (1H, s, OH), 9.99 (1H, s, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.0, 93.1, 115.3, 115.5, 115.8, 117.0, 117.7, 119.4, 130.4, 135.6, 137.6, 157.8, 159.0, 159.6, 160.1, 168.0; *m/z* (EI), 360 (M^+); found 360.068956 ($C_{19}H_{12}N_4O_2S$ M^+, requires 360.068098); Purity: HPLC 1, 94%; HPLC 2, 95%.

*8.3.3. 4-[2-Amino-3,5-dicyano-6-(3-hydroxyphenyl sulfanyl)-pyridin-4-yl]-benzoic acid methyl ester (**17**)*

Yield 27%, yellow powder. Mpt., 224–226 °C, decompose; ν_{max} (solid)/cm^{-1}, 3418.1, 3330.1, 3232.0, 2357.1, 2193.0, 5152.6, 1997.6, 1957.9, 1849.7, 1711.0, 1639.9, 1571.2, 1545.1, 1527.0, 1473.9, 1433.2, 1405.2, 1319.4, 1270.0, 1115.4, 1019.8, 994.7, 889.0; δ_H/ppm (250 MHz, d_6-DMSO), 3.90 (3H, s, CH_3), 6.85–7.10 (3H, m, Ar-H), 7.29 (1H, t, $J = 4.0$, Ar-H), 7.72 (2H, d, $J = 4.0$, Ar-H), 7.91 (2H, bs, NH_2), 8.14 (2H, d, $J = 4.0$, Ar-H), 9.81 (1H, s, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 52.9, 87.4, 93.7, 115.3, 115.6, 117.5, 121.8, 125.8, 128.0, 129.5, 129.9, 130.8, 131.7, 138.9, 158.1, 158.4, 160.1, 166.1, 166.9; *m/z* (EI), 402 (M^+); found 402.078545 ($C_{21}H_{14}N_4O_3S$ M^+, requires 402.078662); Purity: HPLC 1, 97%; HPLC 2, 92%.

*8.3.4. 4-[2-Amino-6-(4-chlorophenylsulfanyl)-3,5-dicyano pyridin-4-yl]-benzoic acid methyl ester (**18**)*

After purification by flash column chromatography (*n*-hexane/ethyl acetate, 2:1), product **18** was isolated as a white powder (52% yield). Mpt., 246–247 °C, decompose; ν_{max} (solid)/cm^{-1}, 3456.8, 3307.8, 3231.3, 2535.7, 2359.8, 2215.5, 2170.0, 2150.0, 2024.4, 1952.1, 1919.9, 1772.0, 1700.1, 1652.6, 1635.9, 1558.1, 1540.4, 1521.0, 1506.7, 1473.0, 1457.5, 1418.3, 1067.2, 1031.4, 977.9, 876.3; δ_H/ppm (250 MHz, d_6-DMSO), 3.91 (3H, s, CH_3), 7.56 (2H, d, $J = 4.5$, Ar-H), 7.63 (2H, d, $J = 4.5$, Ar-H), 7.72 (2H, d, $J = 4.5$, Ar-H), 7.95 (2H, bs, NH_2), 8.14 (2H, d, $J = 4.5$, Ar-H); δ_C/ppm (62.9 MHz, d_6-DMSO), 52.5, 87.0, 114.7, 115.0, 126.0, 129.0, 129.5, 131.3, 134.9, 136.7, 138.4, 157.7, 159.5, 165.6, 165.8, *m/z* (ES), 421 ($[M + H]^+$); found 421.0536 ($C_{21}H_{13}ClN_4O_2S$ $[M + H]^+$, requires 421.0526).

*8.3.5. 4-(2-Amino-6-benzylsulfanyl-3,5-dicyanopyridin-4-yl)-benzoic acid methyl ester (**22**)*

Yield 39%, yellow powder. Mpt., 219–220 °C, decompose; ν_{max} (solid)/cm^{-1}, 3451.7, 3330.9, 3220.2, 2213.7, 2143.3, 1981.0, 1724.4, 1628.8, 1568.4, 1528.1, 1462.7, 1432.5, 1422.4, 1407.3, 1316.7, 1261.4, 1190.9, 1115.4, 1105.4, 1029.9, 964.5, 919.2; δ_H/ppm (250 MHz, d_6-DMSO), 3.89 (3H, s, CH_3), 4.51 (2H, s, CH_2), 7.20–7.40 (3H, m, Ar-H), 7.52 (2H, d, $J = 3.5$, Ar-H), 7.69 (2H, d, $J = 4.0$, Ar-H), 8.10 (2H, d, $J = 4.0$, Ar-H), 8.20 (2H, bs, NH_2); δ_C/ppm (62.9 MHz, d_6-DMSO), 33.7, 52.9, 86.3, 93.4, 115.5, 127.8, 128.9, 129.5, 129.9, 131.7, 138.0, 139.0, 158.0, 159.9, 166.1, 166.8; *m/z* (EI), 400 (M^+); found 400.098441 ($C_{22}H_{16}N_4O_2S$ M^+, requires 400.099398).

*8.3.6. 4-[6-Amino-4-(4-chlorophenyl)-3,5-dicyanopyrid-in-2-ylsulfanyl]-benzoic acid methyl ester (**24**)*

After recrystallisation from THF, product **24** was isolated as a white powder (43% yield). Mpt., 262–263 °C, decompose; ν_{max} (solid)/cm^{-1}, 3459.2, 3403.3, 3283.8, 3232.0, 2364.8, 1636.0, 1558.0, 1540.0, 1521.9, 1274.5, 1180.1, 1059.1, 980.9; δ_H/ppm (250 MHz, d_6-DMSO), 3.89 (3H, s, CH_3), 7.61 (2H, d, $J = 4.5$, Ar-H), 7.68 (2H, d, $J = 4.5$, Ar-H), 7.75 (2H, d, $J = 4.0$, Ar-H), 7.96 (2H, bs, NH_2), 8.03 (2H, d, $J = 4.0$, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.3, 106.8, 128.9, 129.8, 130.4, 134.3; *m/z* (ES), 421 ($[M + H]^+$); found 421.0509 ($C_{21}H_{13}ClN_4SO_2$ $[M + H]^+$, requires 421.0526).

*8.3.7. 4-[6-Amino-3,5-dicyano-4-(3-hydroxyphenyl)-pyridin-2-ylsulfanyl]-benzoic acid methyl ester (**25**)*

Yield 41%, yellow powder. Mpt., 269–270 °C; ν_{max} (solid)/cm^{-1}, 2354.6, 2217.8, 2203.6, 1706.9, 1643.7, 1547.2, 1522.0, 1494.7, 1475.2, 1441.9, 1396.7, 1293.9, 1277.6, 1257.5, 1215.4, 1180.1, 1122.4, 1110.3, 1083.2, 1050.2, 1013.6, 996.5, 951.4, 882.1; δ_H/ppm (250 MHz, d_6-DMSO), 3.89 (3H, s, CH_3), 6.85–7.05 (3H, m, Ar-H), 7.76 (2H, d, $J = 4.0$, Ar-H), 7.88 (2H, bs, NH_2), 8.02 (2H, d, $J = 4.5$, Ar-H), 9.91 (1H, s, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 52.9, 87.9, 94.3, 115.4, 115.5, 115.7, 117.8, 119.4, 126.6, 130.3, 130.5, 130.6, 134.2, 134.8, 135.5, 157.8, 159.3, 160.2, 165.3, 166.3; *m/z* (EI), 402 (M^+); found 402.079337 ($C_{21}H_{14}N_4O_3S$ M^+, requires 402.078662); Purity: HPLC 1, 92%; HPLC 2, 93%.

*8.3.8. 2-Amino-4-(4-chlorophenyl)-6-(4-hydroxyphenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**29**)*

After stirring at room temperature for 18 h, product **30** was isolated as a yellow powder (28% yield). Mpt., 252–253 °C; ν_{max} (solid)/cm^{-1}, 3482.1, 3396.0, 3341.4, 3222.2, 2213.2, 1632.6, 1599.1, 1573.9, 1542.3, 1520.5, 1491.8, 1455.9, 1420.7, 1365.4, 1317.1, 1279.9, 1256.9, 1235.4, 1172.5, 1092.1, 1034.5, 1010.4, 885.8, 831.9, 820.5, 803.9; δ_H/ppm (250 MHz, d_6-DMSO), 6.87 (2H, d, $J = 8.5$, Ar-H), 7.37 (2H, d, $J = 8.5$, Ar-H), 7.59 (2H, d, $J = 8.5$, Ar-H), 7.66 (2H, d, $J = 8.5$, Ar-H), 7.81 (2H, bs, NH_2), 9.99 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 86.7, 92.7, 114.8, 115.0, 115.2, 116.6, 128.9, 130.5, 132.9, 135.3, 137.1, 157.4, 159.2, 159.6, 167.6; *m/z* (EI), 378 (M^+); found 378.035525 ($C_{19}H_{11}ClN_4OS$ M^+, required 378.034211).

*8.3.9. 2-Amino-4-(4-chlorophenyl)-6-(3-hydroxyphenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**30**)*

Yield 29%, yellow powder. Mpt., 213–214 °C, decompose; ν_{max} (solid)/cm^{-1}, 3476.6, 3409.6, 3339.8, 3222.4, 2356.4, 2341.8, 2208.6, 2177.1, 1646.2, 1633.2, 1574.1, 1546.5, 1522.7, 1494.8, 1475.8, 1447.6, 1423.7, 1371.1, 1318.5, 1258.2, 1093.9, 1037.4, 995.5, 890.1, 858.0, 833.3, 806.0; δ_H/ppm (250 MHz, d_6-DMSO), 6.80–7.05 (3H, m, Ar-H), 7.29 (1H, t, $J = 8.0$, Ar-H), 7.62 (2H, d, $J = 4.5$, Ar-H), 7.64 (2H, d, $J = 4.5$, Ar-H), 7.88 (2H, bs, NH_2), 9.80 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 87.1, 93.4, 114.9, 115.2, 117.0, 121.3, 125.3, 127.6, 128.9, 130.3, 130.4, 132.8, 135.3, 157.5, 157.9, 159.6, 166.4; *m/z* (EI), 378 (M^+); found 378.033594 ($C_{19}H_{11}ClN_4OS$ M^+, requires 378.034211).

*8.3.10. 2-Amino-4-(4-chlorophenyl)-6-(4-chlorophenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**31**)*

Yield 37%, yellow powder. Mpt., 261–262 °C; ν_{max} (solid)/cm^{-1}, 3477.9, 3347.4, 3221.7, 2213.8, 2036.2, 1993.3, 1893.9, 1629.4, 1591.8, 1572.0, 1542.1, 1522.6, 1492.2, 1474.0, 1419.9, 1390.0, 1319.2, 1256.8, 1233.5, 1190.2, 1177.0, 1114.0, 1090.3, 1035.4, 1011.8, 953.9, 876.4, 833.0, 821.3; δ_H/ppm (250 MHz, d_6-DMSO), 7.45–7.75 (8H, m, Ar-H), 7.92 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 87.2, 93.2, 114.8, 115.1, 125.9, 128.9, 129.5, 130.4, 132.7, 134.8, 135.4, 136.7, 157.5, 159.6, 165.7; *m/z* (EI), 396 (M^+); found 396.001444 ($C_{19}H_{10}Cl_2N_4S$ M^+, requires 396.000324).

*8.3.11. 3-[6-Amino-4-(4-chlorophenyl)-3,5-dicyano-2-yl sulfanyl]-benzoic acid methyl ester (**32**)*

Yield 41%, yellow powder. Mpt., 250–251 °C, decompose; ν_{max} (solid)/cm^{-1}, 3465.3, 3399.4, 3330.0, 3226.0, 2362.8, 2332.4, 2212.1, 1712.0, 1648.2, 1631.9, 1594.6, 1571.9, 1544.4, 1523.2, 1497.1, 1430.4, 1416.7, 1285.2, 1259.9, 1188.7, 1124.4, 1097.6, 1033.9, 999.8, 977.4, 893.5, 837.9, 805.2; δ_H/ppm (250 MHz, d_6-DMSO), 3.88 (3H, s, CH_3), 7.55–7.75 (5H, m, Ar-H), 7.80–8.00 (3H, m, Ar-H, NH_2), 8.05–8.15 (2H, m, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.4, 87.3, 93.3, 114.9, 115.2, 127.9, 128.9, 130.0, 130.5, 130.8, 132.8, 135.4, 135.6, 139.6, 157.6, 159.6, 165.5, 165.6; *m/z* (EI), 420 (M^+); found 420.043575 ($C_{21}H_{13}ClN_4O_2S$ M^+, requires 420.044775).

*8.3.12. 2-Amino-6-benzylsulfanyl-4-(4-chlorophenyl)-pyridine-3,5-dicarbonitrile (**33**)*

Yield 40%, yellow powder. Mpt., 203–204 °C, decompose; ν_{max} (solid)/cm^{-1}, 3435.1, 3329.2, 3217.6, 2210.9, 2130.3, 1625.7, 1596.0, 1570.3, 1527.4, 1492.9, 1459.7, 1403.4, 1318.9, 1261.5, 1238.1, 1091.2, 1018.1, 917.9, 888.5, 833.4, 801.6; δ_H/ppm (250 MHz, d_6-DMSO), 4.51 (2H, s, CH_2), 7.20–7.40 (3H, m, Ar-H), 7.40–7.70 (6H, m, Ar-H), 8.18 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 33.2, 86.0,

93.2, 115.1, 115.2, 127.3, 128.4, 128.8, 129.4, 130.4, 132.8, 135.3, 137.5, 157.3, 159.5, 166.2; *m/z* (EI), 376 (M^+); found 376.054921 ($C_{20}H_{13}ClN_4S$ M^+, requires 376.054946).

*8.3.13. 2-Amino-6-(4-chloro-6-phenylsulfanyl)-4-(3-hydroxyphenyl)-pyridine-3,5-dicarbonitrile (**35**)*

Yield 13%, yellow powder. Mpt., 187–188 °C, decompose; ν_{max} (solid)/cm^{-1}, 3322.8, 3227.8, 2361.1, 2211.4, 1640.6, 1621.7, 1545.8, 1518.3, 1494.7, 1473.0, 1420.1, 1387.4, 1310.6, 1275.4, 1247.7, 1217.8, 1087.4, 1034.2, 1011.6, 952.5; δ_H/ppm (250 MHz, d_6-DMSO), 6.82–7.05 (3H, m, Ar-H), 7.36 (1H, t, $J = 8.0$, Ar-H), 7.55 (2H, d, $J = 4.5$, Ar-H), 7.62 (2H, d, $J = 4.5$, Ar-H), 7.84 (2H, bs, NH_2), 9.91 (1H, s, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.5, 93.6, 115.4, 115.5, 115.7, 117.8, 119.3, 126.6, 129.9, 130.5, 135.3, 135.5, 137.2, 157.8, 159.1, 160.1, 166.2; *m/z* (EI), 378 (M^+); found 378.033175 ($C_{19}H_{11}ClN_4OS$ M^+, requires 378.034211); Purity: HPLC 1, 93%; HPLC 2, 93%.

*8.3.14. 2-Amino-4-(3-hydroxyphenyl)-6-(3-hydroxy phenylsulfanyl)-pyridine-3,5-dicarbonitrile (**51**)*

Yield 38%, yellow powder. Mpt., 222–223 °C; ν_{max} (solid)/cm^{-1}, 3421.9, 3314.8, 3214.4, 2207.9, 1642.7, 1601.3, 1585.6, 1541.8, 1518.2, 1475.6, 1447.6, 1420.1, 1360.6, 1318.8, 1278.6, 1244.0, 1088.3, 1033.3, 996.1, 950.7, 891.8, 870.1, 845.4; δ_H/ppm (250 MHz, d_6-DMSO), 6.85–7.05 (6H, m, Ar-H), 7.23–7.43 (2H, m, Ar-H), 7.81 (2H, bs, NH_2), 9.85 (2H, bs, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.4, 93.9, 115.5, 115.6, 115.8, 117.4, 119.4, 121.8, 125.8, 128.2, 130.4, 130.8, 135.6, 157.8, 158.4, 159.1, 160.2, 166.2; *m/z* (EI), 360 (M^+); found 360.067480 ($C_{19}H_{12}N_4O_2S$ M^+, requires 360.068098).

*8.3.15. 2-Amino-6-(3-chlorophenylsulfanyl)-4-(3-hydroxy phenyl)-pyridine-3,5-dicarbonitrile (**57**)*

Yield 37%, yellow powder. Mpt., 272–273 °C, decompose; ν_{max} (solid)/cm^{-1}, 3397.9, 3313.8, 3227.8, 2210.2, 2168.1, 1639.7, 1546.3, 1521.9, 1500.2, 1462.8, 1442.5, 1402.6, 1315.8, 1250.9, 1162.0, 1115.3, 1085.5, 1028.4, 998.2, 950.1, 869.0, 843.4; δ_H/ppm (250 MHz, d_6-DMSO), 6.85–7.00 (3H, m, Ar-H), 7.36 (1H, t, $J = 8.0$, Ar-H), 7.45–7.65 (3H, m, Ar-H), 7.69 (1H, s, Ar-H), 7.87 (2H, bs, NH_2), 9.91 (1H, s, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.7, 93.9, 115.4, 115.5, 115.7, 117.8, 119.4, 129.9, 130.2, 130.5, 131.5, 134.0, 134.1, 134.5, 135.5, 157.9, 159.2, 160.2, 165.9; *m/z* (EI), 378 (M^+); found 378.035064 ($C_{19}H_{11}ClN_4OS$ M^+, requires 378.034211).

*8.3.16. 3-[6-Amino-3,5-dicyano-4-(3-hydroxyphenyl)-pyridin-2-ylsulfanyl]-benzoic acid methyl ester (**59**)*

Yield 41%, yellow powder. Mpt., 262–263 °C, decompose; ν_{max} (solid)/cm^{-1}, 3457.8, 3332.7, 3223.3, 2219.3, 1712.9, 1636.7, 1583.1, 1549.3, 1519.0, 1490.2, 1463.6, 1432.2, 1407.1, 1299.7, 1275.7, 1246.8, 1195.4, 1130.7, 1095.9, 1075.4, 1036.1, 998.3, 951.4, 937.8, 882.9, 858.1, 814.0; δ_H/ppm (250 MHz, d_6-DMSO), 3.88 (3H, s, CH_3), 6.85–7.00 (3H, m, Ar-H), 7.36 (1H, t, $J = 8.0$, Ar-H), 7.65 (1H, t, $J = 8.0$, Ar-H), 7.81 (2H, bs, NH_2), 7.88 (1H, d, $J = 7.5$, Ar-H), 8.05–8.15 (2H, m, Ar-H), 9.90 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.4, 87.2, 93.3, 114.9, 115.1, 115.2, 117.3, 118.9, 128.0, 129.9, 130.3, 130.8, 135.0, 135.6, 139.6, 157.4, 158.7, 159.6, 165.5, 165.6; *m/z* (EI), 402 (M^+); found 402.078356 ($C_{21}H_{14}N_4O_3S$ M^+, requires 402.078662).

*8.4. 2-Amino-6-benzylsulfanyl-4-(3,4-dihydroxyphenyl)-pyridine-3,5-dicarbonitrile (**8**)*

To a stirred solution of 3,4-dihydroxybenzaldehyde (2.00 mmol) in ethanol (2.0 mL) containing benzylthiol (2.00 mmol) and malononitrile (0.25 mL, 4.00 mmol) piperidine (0.42 mL, 4.20 mmol) was added. The solution was heated by microwave at 90 °C for 60 min, after which the reaction mixture was dissolved in 4.0 mL ethanol, and stirred in air for 3 h at room temperature. The precipitate formed was collected by suction filtration, washed with *n*-hexane/ethanol (1:1), and then dried under high vacuum. The product was obtained as a yellow powder. Yield 23%; Mpt., 185–186 °C, decompose; ν_{max} (solid)/cm^{-1}, 3460.2, 3343.3, 3208.3, 2208.2, 1621.6, 1602.4, 1519.5, 1493.2, 1398.5, 1348.9, 1317.9, 1281.2, 1268.2, 1248.1, 1120.8, 1027.2, 960.8; δ_H/ppm (250 MHz, d_6-DMSO), 1.41 (6H, t, $J = 6.5$, CH_2), 2.66 (4H, t, $J = 5.5$, CH_2), 4.48 (2H, s, CH_2), 6.78–6.86 (3H, m, Ar-H), 7.25–7.34 (3H, m, Ar-H), 7.49–7.53 (2H, m, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 24.4, 25.9, 33.2, 46.3, 85.6, 93.0, 115.5, 115.7, 120.1, 127.3, 128.4, 129.4, 137.6, 145.7, 158.5, 159.8, 166.2; *m/z* (EI), 374 (M^+), 84 (piperidine); found 374.082130 ($C_{20}H_{14}N_4O_2S$ M^+, requires 374.083748); Purity: HPLC 1, 98%; HPLC 2, 94%.

8.5. General procedure for MWI synthesis in ethanol (3 mmol)

To a stirred solution of aldehyde (3.00 mmol) in ethanol (2.0 mL) containing thiol (3.00 mmol) and malononitrile (0.38 mL, 6.00 mmol) piperidine (0.03 mL, 0.30 mmol) was added. The solution was heated by microwave at 90 °C for 60 min, after which the reaction mixture was dissolved in 7 mL ethanol, exposed to air, and stirred for 3 h at room temperature. The precipitate formed was collected by suction filtration, washed with *n*-hexane/ethanol (1:1).

*8.5.1. 4-[2-Amino-3,5-dicyano-6-(2-hydroxyphenyl-sulfanyl)-pyridin-4-yl]-benzoic acid methyl ester (**23**)*

Yield 42%, yellow powder. Mpt., 262–263 °C; ν_{max} (solid)/cm^{-1}, 3396.1, 3316.4, 3227.2, 2226.0, 1715.4, 1643.0, 1574.2, 1545.3, 1524.0, 1444.4, 1435.3, 1361.4, 1323.9, 1293.8, 1261.3, 1194.5, 1122.8, 1113.4, 1029.8, 969.9; δ_H/ppm (250 MHz, d_6-DMSO), 3.92 (3H, s, CH_3), 6.89 (1H, t, $J = 7.5$, Ar-H), 7.00 (1H, d, $J = 4.5$, Ar-H), 7.35 (1H, t, $J = 8.0$, Ar-H), 7.45 (1H, d, $J = 4.0$, Ar-H), 7.73 (2H, d, $J = 4.0$, Ar-H), 7.81 (2H, bs, NH_2), 8.14 (2H, d, $J = 4.0$, Ar-H), 10.13 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.5, 86.3, 93.0, 112.4, 114.9, 115.3, 116.2, 119.7, 129.1, 129.5, 131.2, 131.9, 136.7, 138.6, 157.5, 158.5, 159.5, 165.7,

166.9; *m/z* (ES), 403 ($[M+H]^+$); found 403.0853 ($C_{21}H_{14}N_4O_3S$ $[M+H]^+$, requires 403.0865).

*8.5.2. 4-[6-Amino-3,5-dicyano-4-(2,3-dihydrobenzo [1,4]dioxin-6-yl)-pyridin-2-ylsulfanyl]-benzoic acid methyl ester (**28**)*

Yield 44%, yellow powder. Mpt., 257–258 °C, decompose; ν_{max} (solid)/cm^{-1}, 3410.8, 3326.3, 3228.4, 2215.4, 1706.8, 1643.2, 1549.4, 1529.4, 1507.8, 1435.7, 1276.8, 1251.9, 1198.0, 1110.8, 1063.2, 1013.6; δ_H/ppm (250 MHz, d_6-DMSO), 3.89 (3H, s, CH_3), 4.33 (4H, s, CH_2), 7.04–7.11 (3H, m, Ar-H), 7.75 (2H, d, $J=4.0$, Ar-H), 7.84 (2H, bs, NH_2), 8.02 (2H, d, $J=4.0$, Ar-H); δ_C/ppm (62.9 MHz, d_6-DMSO), 52.9, 64.5, 64.7, 99.0, 99.3, 115.4, 115.9, 117.9, 118.0, 119.0, 130.3, 134.8, 143.7, 145.8, 154.8, 155.8, 158.5, 160.2; *m/z* (EI), 444 (M^+); found 444.089436 ($C_{23}H_{16}N_4O_4S$ M^+, requires 444.089277).

*8.5.3. 2-Amino-4-(4-chlorophenyl)-6-(2-hydroxyphenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**34**)*

Yield 43%, yellow powder. Mpt., 268–269 °C; ν_{max} (solid)/cm^{-1}, 3432.3, 3337.9, 3239.5, 2362.6, 2213.9, 1642.4, 1595.0, 1573.8, 1544.3, 1516.1, 1496.8, 1466.0, 1422.1, 1326.3, 1295.8, 1262.0, 1236.4, 1177.3, 1151.4, 1124.2, 1093.2, 1024.9; δ_H/ppm (250 MHz, d_6-DMSO), 6.88 (1H, t, $J=7.5$, Ar-H), 6.99 (1H, d, $J=4.0$, Ar-H), 7.34 (1H, t, $J=8.0$, Ar-H), 7.44 (1H, d, $J=4.5$, Ar-H), 7.60 (2H, d, $J=4.5$, Ar-H), 7.68 (2H, d, $J=4.5$, Ar-H), 7.77 (2H, bs, NH_2), 10.11 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 86.5, 93.2, 112.5, 115.0, 115.4, 116.2, 119.7, 128.9, 130.5, 131.9, 132.9, 135.3, 136.7, 157.3, 158.5, 159.5, 166.9; *m/z* (EI), 378 (M^+); found 378.033438 ($C_{19}H_{11}ClN_4OS$ M^+, requires 378.034211).

*8.5.4. 2-Amino-6-(4-chlorophenylsulfanyl)-4-(2,3-dihydro benzo[1,4]dioxin-6-yl)-pyridine-3,5-dicarbonitrile (**38**)*

Yield 40%, yellow powder. Mpt., 243–244 °C; ν_{max} (solid)/cm^{-1}, 3477.2, 3331.2, 3221.6, 2222.1, 1635.6, 1587.1, 1546.2, 1504.1, 1473.9, 1433.8, 1386.8, 1311.3, 1287.2, 1251.9, 1177.1, 1131.4, 1090.7, 1066.2; δ_H/ppm (250 MHz, d_6-DMSO), 4.33 (4H, s, CH_2-CH_2), 7.03–7.10 (3H, m, Ar-H), 7.55 (2H, d, $J=4.5$, Ar-H), 7.62 (2H, d, $J=4.0$, Ar-H), 7.81 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 87.1, 93.3, 115.2, 115.4, 117.4, 117.5, 121.8, 126.1, 126.5, 129.4, 134.8, 136.7, 143.2, 145.3, 157.9, 159.7, 165.7; *m/z* (EI), 420 (M^+); found 420.044837 ($C_{21}H_{13}ClN_4O_2S$ M^+, requires 420.044775).

*8.5.5. 2-Amino-4-benzo[1,3]dioxin-5-yl-6-(4-chloro phenylsulfanyl)-pyridine-3,5-dicarbonitrile (**39**)*

Yield 46%, yellow powder. Mpt., 251–252 °C, decomposed; ν_{max} (solid)/cm^{-1}, 3334.0, 2211.2, 1642.8, 1620.6, 1547.4, 1500.0, 1446.7, 1250.6, 1090.1, 1039.2, 1011.4, 924.0, 814.1; δ_H/ppm (250 MHz, d_6-DMSO), 6.16 (2H, s, CH_2), 7.02–7.17 (3H, m, Ar-H), 7.55 (2H, d, $J=4.5$, Ar-H), 7.62 (2H, d, $J=4.5$, Ar-H), 7.83 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 87.3, 93.4, 101.8, 108.6, 108.9, 115.1, 115.4, 123.1, 126.1, 127.3, 129.5, 134.8, 136.7, 147.4, 149.0, 158.2, 159.7, 165.6; *m/z* (EI), 406 (M^+); found 406.029091 ($C_{20}H_{11}ClN_4O_2S$ M^+, requires 406.029125).

*8.5.6. 2-Amino-4-benzo[1,3]dioxol-5-yl-6-(4-hydroxyphenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**40**)*

Yield 31%, yellow powder. Mpt., 268–269 °C, decompose; ν_{max} (solid)/cm^{-1}, 3338.5, 3224.0, 2218.4, 1625.3, 1598.1, 1578.9, 1549.0, 1522.4, 1487.1, 1448.0, 1420.4, 1341.6, 1276.2, 1245.7, 1191.4, 1166.7, 1095.2, 1030.8; δ_H/ppm (250 MHz, d_6-DMSO), 6.15 (2H, s, CH_2), 6.87 (2H, d, $J=4.5$, Ar-H), 7.01–7.17 (3H, m, Ar-H), 7.37 (2H, d, $J=4.5$, Ar-H), 7.72 (2H, bs, NH_2), 9.99 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 87.3, 93.4, 102.3, 109.0, 109.4, 115.4, 115.7, 115.9, 117.0, 123.5, 127.9, 137.6, 147.8, 149.4, 158.5, 159.6, 160.1, 167.9; *m/z* (ES), 389 ($[M+H]^+$); found 389.0716 ($C_{20}H_{12}N_4O_3S$ $[M+H]^+$, requires 389.0708).

*8.5.7. 2-Amino-4-benzo[1,3]dioxol-5-yl-6-(3-hydroxy phenylsulfanyl)-pyridine-3,5-dicarbonitrile (**41**)*

Yield 34%, yellow powder. Mpt., 252–253 °C, decompose; ν_{max} (solid)/cm^{-1}, 3446.3, 3347.8, 3230.3, 2359.5, 2213.5, 1635.9, 1545.8, 1520.9, 1501.3, 1489.6, 1465.7, 1446.0, 1404.1, 1354.4, 1300.2, 1244.6, 1198.5, 1103.3, 1035.5, 994.7, 931.5; δ_H/ppm (250 MHz, d_6-DMSO), 6.16 (2H, s, CH_2), 6.88 (1H, d, $J=4.0$, Ar-H), 6.96–7.18 (5H, m, Ar-H), 7.29 (1H, t, $J=8.0$, Ar-H), 7.79 (2H, bs, NH_2), 9.79 (1H, s, OH); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.7, 94.1, 109.0, 109.5, 115.6, 115.9, 117.4, 121.8, 123.5, 125.8, 127.8, 128.2, 130.8, 147.8, 149.4, 158.4, 158.7, 160.2, 166.7; *m/z* (EI), 388 (M^+); found 388.064023 ($C_{20}H_{12}N_4O_3S$ M^+, requires 388.063012).

*8.5.8. 2-Amino-4-benzo[1,3]dioxin-5-yl-6-(3-chlorophenyl sulfanyl)-pyridine-3,5-dicarbonitrile (**42**)*

Yield 39%, yellow powder. Mpt., 172–173 °C, decompose; ν_{max} (solid)/cm^{-1}, 3318.1, 3225.8, 2215.7, 1637.8, 1550.1, 1522.2, 1498.5, 1447.1, 1407.6, 1352.2, 1252.4, 1105.5, 1035.8; δ_H/ppm (250 MHz, d_6-DMSO), 6.16 (2H, s, CH_2), 7.03–7.17 (3H, m, Ar-H), 7.54–7.56 (3H, m, Ar-H), 7.68 (1H, s, Ar-H), 7.86 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 87.9, 94.1, 102.3, 109.1, 109.4, 115.6, 115.8, 123.5, 127.7, 129.9, 130.2, 131.5, 134.0, 134.1, 134.5, 147.9, 149.5, 158.7, 160.2, 165.9; *m/z* (EI), 406 (M^+); found 406.031081 ($C_{20}H_{11}ClN_4O_2S$ M^+, requires 406.029125).

*8.5.9. 3-(6-Amino-4-benzo[1,3]dioxol-5-yl-3,5-dicyano pyridi-2-ylsulfanyl)-benzoic acid methyl ester (**43**)*

Yield 50%, yellow powder. Mpt., 245–246 °C, decompose; ν_{max} (solid)/cm^{-1}, 3433.7, 3318.3, 3226.4, 2360.1, 2214.6, 1728.2, 1703.2, 1638.3, 1550.5, 1522.0, 1497.3, 1447.6, 1352.6, 1297.3, 1253.1, 1129.7, 1037.6; δ_H/ppm (250 MHz, d_6-DMSO), 3.88 (3H, s, CH_3), 6.16 (2H, s, CH_2), 7.03–7.18 (3H, m, Ar-H), 7.65 (1H, t, $J=8.0$, Ar-H), 7.80 (2H, bs, NH_2), 7.87 (1H, d, $J=4.0$, Ar-H), 8.07 (2H, d, $J=3.0$, Ar-H); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.7, 94.0, 102.3, 109.0, 109.4, 115.6, 115.9, 123.5, 127.7, 127.8, 129.9, 130.2, 135.3, 147.8, 149.5, 158.7, 160.2, 166.5; *m/z* (EI), 430

(M^+); found 430.073462 ($C_{22}H_{14}N_4O_4S$ M^+, requires 430.073577).

*8.5.10. 2-Amino-4-benzo[1,3]dioxol-5-yl-6-benzylsulfanyl-pyridine-3,5-dicarbonitrile (**44**)*

Yield 46%, yellow powder. Mpt., 221–222 °C; ν_{max} (solid)/cm^{-1}, 3471.1, 3329.4, 3213.3, 2217.7, 1616.1, 1547.1, 1526.6, 1497.5, 1485.7, 1440.9, 1407.5, 1349.6, 1311.7, 1248.3, 1233.0, 1194.2, 1123.7, 1106.2, 1030.8; δ_H/ppm (250 MHz, d_6-DMSO), 4.49 (2H, s, CH_2), 6.14 (2H, s, CH_2), 6.99–7.14 (3H, m, Ar-H), 7.22–7.35 (3H, m, Ar-H), 7.51 (2H, d, $J = 3.5$, Ar-H), 8.11 (2H, bs, NH_2); δ_C/ppm (62.9 MHz, d_6-DMSO), 86.6, 93.8, 108.9, 109.4, 115.8, 123.5, 127.7, 127.8, 128.9, 129.9, 138.1, 147.8, 149.4, 158.5, 160.0, 166.6; *m/z* (EI), 386 (M^+); found 386.083988 ($C_{21}H_{14}N_4O_2S$ M^+, requires 386.083748).

*8.5.11. 2-Amino-4-benzo[1,3]dioxol-5-yl-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (**45**)*

Yield 39%, yellow powder. Mpt., 268–269 °C, decompose; ν_{max} (solid)/cm^{-1}, 3450.9, 3332.9, 3229.4, 2214.3, 1633.9, 1551.6, 1526.0, 1489.9, 1450.0, 1408.4, 1346.5, 1310.0, 1246.5, 1173.8, 1035.6, 925.5; δ_H/ppm (250 MHz, d_6-DMSO), 6.16 (2H, s, CH_2), 7.03–7.18 (3H, m, Ar-H), 7.50–7.61 (5H, m, Ar-H), 7.79 (2H, bs, NH_2); δ_C/ppm (62.9 MHz, d_6-DMSO), 87.7, 94.0, 102.3, 109.0, 109.4, 115.6, 115.9, 123.5, 127.7, 127.8, 129.9, 130.2, 135.3, 147.8, 149.5, 158.7, 160.2, 166.5; *m/z* (EI), 372 (M^+); found 372.068906 ($C_{20}H_{12}N_4O_2S$ M^+, requires 372.068098).

*8.5.12. 2-Amino-6-(2-hydroxyphenylsulfanyl)-4-thio-phen-2-yl-pyridine-3,5-dicarbonitrile (**46**)*

Yield 30%, yellow powder. Mpt., 212–213 °C, decompose; ν_{max} (solid)/cm^{-1}, 3439.2, 3341.7, 3236.0, 2211.1, 1638.3, 1543.7, 1512.8, 1468.1, 1433.9, 1404.1, 1325.9, 1298.5, 1260.2, 1234.0, 1100.0; δ_H/ppm (250 MHz, d_6-DMSO), 6.88 (1H, t, $J = 7.5$, Ar-H), 6.98 (1H, d, $J = 4.0$, Ar-H), 7.28–7.37 (2H, m, Ar-H), 7.43 (1H, d, $J = 3.5$, Ar-H), 7.58 (1H, d, $J = 2.0$, Ar-H), 7.76 (2H, bs, NH_2), 7.96 (1H, d, $J = 2.5$, Ar-H), 10.10 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 86.3, 93.1, 112.5, 115.3, 115.6, 116.2, 119.7, 127.9, 130.7, 131.2, 131.9, 132.8, 136.7, 150.7, 158.5, 159.7, 167.4; *m/z* (EI), 350 (M^+); found 350.030999 ($C_{17}H_{10}N_4OS_2$ M^+, requires 350.029605).

*8.5.13. 2-Amino-4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-6-(2-hydroxyphenylsulfanyl)-pyridine-3,5-dicarbonitrile (**47**)*

Yield 20%, yellow powder. Mpt., 150–151 °C; ν_{max} (solid)/cm^{-1}, 3417.2, 3334.6, 3239.8, 2213.4, 1648.8, 1586.7, 1546.3, 1505.9, 1470.6, 1432.1, 1309.8, 1286.0, 1254.1, 1178.6, 1128.9, 1068.4; δ_H/ppm (250 MHz, d_6-DMSO), 4.33 (4H, s, CH_2-CH_2), 6.88 (1H, t, $J = 7.5$, Ar-H), 6.97–7.10 (4H, m, Ar-H), 7.34 (1H, t, $J = 7.5$, Ar-H), 7.43 (1H, d, $J = 4.0$, Ar-H), 7.66 (2H, bs, NH_2), 10.09 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 66.6, 86.4, 93.3, 112.6, 115.3, 115.7, 116.2, 117.4, 117.5, 119.7, 121.8, 126.6, 131.8, 136.7, 143.2, 145.2, 157.6, 158.5, 159.7, 166.9; *m/z* (EI), 402 (M^+); found 402.078829 ($C_{21}H_{14}SN_4O_4$ M^+, requires 402.078662).

*8.5.14. 2-Amino-6-(3-chlorophenylsulfanyl)-4-(2,3-dihydro benzo[1,4]dioxin-6-yl)-pyridine-3,5-dicarbonitrile (**52**)*

Yield 34%, yellow powder. Mpt., 200–201 °C; ν_{max} (solid)/cm^{-1}, 3330.5, 2208.9, 2150.2, 2072.2, 2042.0, 1991.5, 1617.9, 1583.9, 1545.4, 1507.5, 1460.5, 1429.8, 1411.4, 1324.0, 1294.4, 1251.8, 1198.7, 1124.1, 1069.6, 1040.9; δ_H/ppm (250 MHz, d_6-DMSO), 4.33 (4H, s, CH_2-CH_2), 7.02–7.11 (3H, m, Ar-H), 7.51–7.60 (3H, m, Ar-H), 7.68 (1H, t, $J = 1.5$, Ar-H), 7.84 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 87.3, 93.5, 115.2, 115.4, 117.4, 117.5, 121.8, 126.5, 129.5, 129.7, 131.0, 133.5, 133.6, 134.0, 143.2, 145.3, 157.9, 159.8, 165.5; *m/z* (EI), 420 (M^+); found 420.043819 ($C_{21}H_{13}ClN_4O_2S$ M^+, requires 420.044775).

*8.5.15. 3-[6-Amino-3,5-dicyano-4-(2,3-dihydrobenzo[1,4] dioxin-6-yl)-pyridin-2-ylsulfanyl]-benzoic acid methyl ester (**53**)*

Yield 43%, yellow powder. Mpt., 251–252 °C, decompose; ν_{max} (solid)/cm^{-1}, 333.1, 3231.7, 2197.6, 1730.0, 1708.5, 1649.1, 1585.6, 1548.3, 1504.9, 1434.4, 1310.1, 1251.0, 1125.8, 1067.3, 915.9, 887.3; δ_H/ppm (250 MHz, d_6-DMSO), 3.88 (3H, s, CH_3), 4.33 (4H, s, CH_2), 7.04 (2H, s, Ar-H), 7.11 (1H, s, Ar-H), 7.65 (1H, t, $J = 8.0$, Ar-H), 7.79 (2H, bs, NH_2), 7.88 (1H, d, $J = 4.0$, Ar-H), 8.07 (2H, d, $J = 3.0$, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.4, 64.1, 64.3, 87.2, 93.4, 115.2, 115.4, 117.4, 117.5, 121.8, 126.5, 128.1, 130.0, 130.3, 130.8, 135.6, 139.6, 143.2, 145.3, 157.9, 159.7, 165.5, 165.6; *m/z* (EI), 444 (M^+); found 444.090396 ($C_{23}H_{16}N_4O_4S$ M^+, requires 444.089277).

*8.5.16. 2-Amino-4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-6-(4-hydroxyphenylsulfanyl)-pyridine-3,5-dicarbonitrile (**54**)*

Yield 35%, yellow powder. Mpt., 221–222 °C, decompose; ν_{max} (solid)/cm^{-1}, 3352.5, 3290.3, 3180.1, 2213.7, 1629.5, 1581.7, 1532.9, 1497.8, 1453.8, 1428.1, 1286.6, 1249.7, 1201.0, 1169.6, 1131.3, 1057.9; δ_H/ppm (250 MHz, d_6-DMSO), 4.32 (4H, s, CH_2-CH_2), 6.86 (2H, d, $J = 4.0$, Ar-H), 7.01–7.09 (3H, m, Ar-H), 7.37 (2H, d, $J = 4.5$, Ar-H), 7.70 (2H, bs, NH_2), 9.99 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 86.6, 92.7, 114.9, 115.3, 115.5, 116.5, 117.4, 117.5, 121.8, 126.6, 137.1, 143.2, 145.2, 157.7, 159.1, 159.7, 167.6; *m/z* (ES), 403 ($[M + H]^+$); found 403.0879 ($C_{21}H_{14}N_4O_3S$ $[M + H]^+$, requires 403.0865).

*8.5.17. 2-Amino-6-(3-hydroxyphenylsulfanyl)-4-thiophen-2-yl-pyridine-3,5-dicarbonitrile (**55**)*

Yield 28%, yellow powder. Mpt., 232–233 °C; ν_{max} (solid)/cm^{-1}, 3379.2, 3316.3, 3220.2, 2209.0, 1645.7, 1541.0, 1507.5, 1441.2, 1247.4, 1223.4, 1078.3, 1012.7, 995.5, 885.1, 851.9; δ_H/ppm (250 MHz, d_6-DMSO), 6.87–7.02 (3H, m, Ar-H); 7.26–7.31 (2H, m, Ar-H); 7.58 (1H, d, $J = 2.0$, Ar-H); 7.86 (2H, bs, NH_2); 7.96 (1H, d, $J = 2.5$, Ar-H); 9.80 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 86.8, 115.1, 115.4, 117.0, 121.3, 125.3, 127.6, 127.9, 130.3, 130.8, 131.3,

132.7, 150.9, 157.9, 159.9, 166.9; *m/z* (ES), 351 (M^+); found 351.0388 ($C_{17}H_{10}N_4S_2O$ $[M+H]^+$, requires 351.0374); Purity, HPLC 1, 76%; HPLC 2, 79%.

*8.5.18. 2-Amino-4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-6-(3-hydroxyphenylsulfanyl)-pyridine-3,5-dicarbonitrile (**56**)*

Yield 46%, yellow powder. Mpt., 236–237 °C, decompose; ν_{max} (solid)/cm^{-1}, 3438.1, 3330.1, 3218.1, 2212.5, 1629.2, 1581.9, 1545.6, 1502.8, 1432.0, 1305.7, 1285.0, 1249.8, 1199.9, 1126.8, 1065.7, 995.5, 919.0, 887.4; δ_H/ppm (250 MHz, d_6-DMSO), 4.33 (4H, s, CH_2-CH_2), 6.88 (1H, d, $J = 4.5$, Ar-H), 6.96–7.11 (5H, m, Ar-H), 7.28 (1H, t, $J = 7.5$, Ar-H), 7.77 (2H, bs, NH_2), 9.79 (1H, s, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 87.0, 93.5, 115.2, 115.5, 116.9, 117.4, 117.5, 121.3, 121.8, 125.3, 126.6, 127.8, 130.3, 143.2, 145.2, 157.9, 159.8, 166.3; *m/z* (EI), 402 (M^+); found 402.07932 ($C_{21}H_{14}N_4O_3S$ M^+, requires 402.078662).

*8.5.19. 2-Amino-6-benzylsulfanyl-4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-pyridine-3,5-dicarbonitrile (**62**)*

Yield 40%, yellow powder. Mpt., 195–196 °C; ν_{max} (solid)/cm^{-1}, 3442.0, 3326.6, 3221.0, 2360.1, 2208.4, 1629.9, 1584.8, 1543.2, 1505.9, 1458.6, 1431.1, 1287.6, 1250.5, 1199.4, 1130.2, 1067.1; δ_H/ppm (250 MHz, d_6-DMSO), 4.30 (4H, s, CH_2-CH_2), 4.49 (2H, s, CH_2), 6.99–7.07 (3H, m, Ar-H), 7.25–7.34 (3H, m, Ar-H), 7.51 (2H, d, $J = 4.0$, Ar-H), 8.10 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 85.9, 93.2, 115.4, 117.3, 117.5, 121.8, 126.6, 127.3, 128.4, 129.4, 137.6, 143.2, 145.2, 157.7, 159.6, 166.2; *m/z* (ES), 401 ($[M+H]^+$); found 401.1075 ($C_{22}H_{16}N_4O_2S$ $[M+H]^+$, requires 401.1072).

*8.5.20. 2-Amino-4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-6-phenylsulfanyl-pyridine-3,5-dicarbonitrile (**63**)*

Yield 29%, yellow powder. Mpt., 227–228 °C; ν_{max} (solid)/cm^{-1}, 3444.4, 3340.7, 3228.6, 2360.7, 2217.7, 1640.8, 1582.2, 1545.5, 1504.2, 1433.2, 1308.6, 1285.6, 1249.8, 1129.8, 1066.6; δ_H/ppm (250 MHz, d_6-DMSO), 4.33 (4H, s, CH_2-CH_2), 7.03–7.10 (3H, m, Ar-H), 7.50–7.59 (5H, m, Ar-H), 7.76 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 64.1, 64.3, 87.0, 93.4, 115.2, 115.5, 117.4, 117.5, 121.8, 126.6, 127.2, 129.4, 129.7, 134.8, 143.2, 145.3, 157.9, 159.8, 166.1; *m/z* (ES), 387 ($[M+H]^+$); found 387.0897 ($C_{21}H_{14}N_4O_4S$ $[M+H]^+$, requires 387.0916).

8.6. General procedure for the synthesis of 4-furan-2-yl-pyridine-3,5-dicarbonitriles

Malononitrile (0.132 g, 2.0 mmol), thiol (4.0 mmol) and piperidine (1 drop) were dissolved in ethanol (8 mL) and refluxed for 30 min. 2-Furan-2-ylmethylenemalononitrile **13** (0.288 g, 2.0 mmol) was added as a solid to the hot reaction mixture and reflux was maintained for further 30 min. After allowing the reaction mixture to cool down it was exposed to air over night. The solids formed were collected, washed twice with ethanol/water (10 mL, 1:1) and dried under high vacuum. If no solids were formed water (1 mL) was added and the mixture was allowed to stand for 2 h. This was repeated until formation of solids was observed. Solids were isolated as above.

*8.6.1. 4-(6-Amino-3,5-dicyano-4-furan-2-ylpyridin-2-ylsulfanyl)-benzoic acid methyl ester (**27**)*

Yield 6%, pale orange solid. Mpt., 267–268 °C decompose; ν_{max} (Solid)/cm^{-1}, 3401, 3326, 3227, 2211, 1706, 1645, 1538, 1517, 1267, 1117, 1012, 758; δ_H/ppm (250 MHz, d_6-DMSO), 3.89 (3H, s, CH_3), 6.85 (1H, dd, $J = 4.0$, 2.0, Ar-H), 7.43 (1H, d, $J = 3.5$, Ar-H), 7.74 (2H, d, $J = 8.5$, Ar-H), 7.89 (2H, bs, NH_2), 8.01 (2H, d, $J = 8.5$, Ar-H), 8.13 (1H, d, $J = 1.0$, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.6, 83.2, 113.1, 115.6, 115.8, 116.8, 126.4, 130.0, 130.3, 133.9, 134.6, 144.3, 145.2, 155.3, 160.4, 165.9, 166.3; *m/z* (EI), 376 (M^+); found 376.0612 ($C_{19}H_{12}N_4O_3S$ M^+, requires 376.0630); Purity, HPLC 1, 93%; HPLC 2, 93%.

*8.6.2. 2-Amino-6-(4-chlorophenylsulfanyl)-4-furan-2-yl-pyridine-3,5-dicarbonitrile (**3**)*

Yield 14%, pale yellow solid. Mpt., 276–278 °C, decompose; ν_{max} (Solid)/cm^{-1}, 3480, 3319, 3212, 2212, 1624, 1571, 1541, 1513, 1470, 1268, 1240, 1010, 828, 760; δ_H/ppm (250 MHz, d_6-DMSO), 6.85 (1H, dd, $J = 3.5$, 1.5, Ar-H), 7.42 (1H, dd, $J = 3.5$, 0.6, Ar-H), 7.53 (2H, dt, $J = 8.5$, 2.0, Ar-H), 7.61 (2H, dt, $J = 9.0$, 2.0, Ar-H), 7.84 (2H, bs, NH_2), 8.12 (1H, dd, $J = 2.0$, 0.5, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 82.9, 89.4, 113.1, 115.7, 115.9, 116.7, 126.3, 129.6, 134.9, 136.9, 138.1, 144.2, 145.2, 160.4, 167.1; *m/z* (ES), 353 ($[M+H]^+$); found 353.0249 ($C_{17}H_{10}ClN_4OS$ $[M+H]^+$, requires 353.0264).

*8.6.3. 2-Amino-4-furan-2-yl-6-(4-hydroxyphenylsulfanyl)-pyridine-3,5-dicarbonitrile (**48**)*

After addition of water (7 × 1.0 mL). Yield 7%, yellow solid. Mpt., 240–241 °C; ν_{max} (Solid)/cm^{-1}, 3332, 3230, 2217, 1630, 1536, 1508, 1476, 1412, 1268, 1227, 1168, 1024, 833, 765; δ_H/ppm (250 MHz, d_6-DMSO), 6.82–6.90 (3H, m, Ar-H), 7.32–7.42 (3H, m, Ar-H), 7.74 (2H, bs, NH_2), 8.1 (1H, dd, $J = 1.5$, 0.5, Ar-H), 9.99 (1H, bs, OH); δ_C/ppm (62.8 MHz, d_6-DMSO), 82.5, 89.0, 113.0, 115.1, 115.8, 115.9, 116.5, 116.7, 137.3, 144.1, 145.3, 159.3, 160.4, 168.9; *m/z* (ES), 333 ($[M-H]^-$); found 333.0460 ($C_{17}H_9N_4O_2S$ $[M-H]^-$, requires 333.0446).

*8.6.4. 3-(6-Amino-3,5-dicyano-4-furan-2-ylpyridin-2-ylsulfanyl)-benzoic acid methyl ester (**49**)*

Yield 16%, pale orange solid. Mpt., 269–271 °C decompose; ν_{max} (solid)/cm^{-1}, 3468, 3358, 2213, 1724, 1630, 1522, 1440, 1266, 1127, 1022, 768, 742; δ_H/ppm (250 MHz, d_6-DMSO), 3.87 (3H, s, CH_3), 6.80–6.90 (1H, m, Ar-H), 7.38–7.46 (1H, m, Ar-H), 7.60–7.70 (1H, m, Ar-H), 7.76–7.92 (3H, m, Ar-H + NH_2), 8.02–8.10 (2H, m, Ar-H), 8.11–8.16 (1H, m, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 52.6, 83.0, 89.5, 113.1, 115.7, 115.9, 116.7, 118.7, 128.2, 130.1, 130.5, 130.9, 135.8, 139.9, 144.2, 145.2, 160.4, 165.6, 186.3; *m/z* (EI), 376 (M^+); found 376.0612 ($C_{19}H_{12}N_4O_3S$ M^+, requires 376.0630).

*8.6.5. 2-Amino-6-benzylsulfanyl-4-furan-2-yl-pyridine-3,5-dicarbonitrile (**50**)*

Yield 6%, yellow solid. Mpt., 219–220 °C, decompose; ν_{max} (solid)/cm^{-1}, 3444, 3324, 3211, 2208, 1622, 1537, 1515, 1414, 1240, 1025, 757, 698; δ_H/ppm (250 MHz, d_6-DMSO), 4.48 (2H, s, CH_2), 6.82 (1H, dd, $J = 3.5$, 2.0, Ar-H), 7.20–7.40 (4H, m, Ar-H), 7.46–7.54 (2H, m, Ar-H), 8.09 (1H, dd, $J = 2.0$, 0.5, Ar-H), 8.11 (2H, bs, NH_2); δ_C/ppm (62.8 MHz, d_6-DMSO), 33.5, 81.8, 89.4, 104.2, 113.0, 115.9, 116.5, 127.5, 128.6, 129.5, 137.6, 144.0, 145.3, 146.7, 160.3, 167.5; *m/z* (ES), 333 ($[M + H]^+$); found 333.0820 ($C_{18}H_{13}N_4OS$ $[M + H]^+$, requires 333.0810).

*8.6.6. 2-Amino-6-(3-chlorophenylsulfanyl)-4-furan-2-yl-pyridine-3,5-dicarbonitrile (**58**)*

After addition of water (3 × 1.0 mL). Yield 9%, off white solid. Mpt., 198–200 °C; ν_{max} (Solid)/cm^{-1}, 3468, 3312, 3210, 2206, 1622, 1535, 1511, 1459, 1397, 1266, 1023, 775, 757; δ_H/ppm (250 MHz, d_6-DMSO), 6.85 (1H, dd, $J = 3.5$, 2.0, Ar-H), 7.42 (1H, d, $J = 3.5$, Ar-H), 7.46–7.60 (3H, m, Ar-H), 7.67 (1H, t, $J = 1.5$, Ar-H), 7.87 (2H, bs, NH_2), 8.13 (1H, d, $J = 1.5$, Ar-H); δ_C/ppm (62.8 MHz, d_6-DMSO), 83.0, 89.6, 113.0, 115.6, 115.8, 116.7, 129.6, 129.9, 131.1, 133.7, 134.3, 143.9, 144.2, 145.2, 146.8, 160.4, 166.9; *m/z* (ES), 353 ($[M + H]^+$); found 353.0271 ($C_{17}H_{10}ClN_4OS$ $[M + H]^+$, requires 353.0264).

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejmech.2007.02.018.

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