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Inhibition of the pore-forming protein perforin by a series of aryl-substituted isobenzofuran-1(3*H*)-ones

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ABSTRACT

An aryl-substituted isobenzofuran-1(3*H*)-one lead compound was identified from a high throughput screen designed to find inhibitors of the lymphocyte pore-forming protein perforin. A series of analogs were then designed and prepared, exploring structure–activity relationships through variation of 2-thioxoimidazolidin-4-one and furan subunits on an isobenzofuranone core. The ability of the resulting compounds to inhibit the lytic activity of both isolated perforin protein and perforin delivered in situ by intact KHYG-1 natural killer effector cells was determined. Several compounds showed excellent activity at concentrations that were non-toxic to the killer cells. This series represents a significant improvement on previous classes of compounds, being substantially more potent and largely retaining activity in the presence of serum.

1. Introduction

The granule exocytosis pathway is the principal mechanism employed by natural killer (NK) cells and cytotoxic T lymphocytes (CTL) to eliminate virus-infected and transformed cells.[1–3] These key effector cells contain secretory vesicles (granules) which are used to store various cytotoxic proteins, including a group of pro-apoptotic serine proteases known as granzymes,[4,5] and perforin, a 67 kDa calcium-dependent pore-forming glycoprotein.[6–10] CTL and NK cells are protected from the inherent cytotoxicity of perforin during synthesis, trafficking and storage by a variety of mechanisms,[11] including the low pH present in the secretory granules (typically 5.1–5.5).[12] This results in protonation of key calcium-binding aspartate residues, rendering perforin incapable of calcium-triggered activation. Stable conjugation of the cytotoxic effector cell with a target cell results in migration of granules to the site of contact, followed by exocytic delivery of the granule contents into the immune synapse. Exposure to this neutral, calcium-rich environment then allows perforin monomers to coordinate calcium and oligomerize into highly ordered aggregates of 19–24 subunits that form trans-membrane pores, 130–200 Å in diameter, which penetrate the plasma membrane.[13] This process facilitates entry of the granzymes into the cytosol of the infected or transformed cell where they initiate various apoptotic death mechanisms.[4]

The membrane-attack complex/perforin (MACPF) domain that is responsible for lytic activity in perforin is shared by a lytic protein toxin from *Photorhabdus luminescens* (Plu-MACPF), for which the crystal structure has been published.[14] This 2.0 Å resolution structure revealed similarities with pore-forming cholesterol-dependent cytolysins (CDCs) from gram-positive bacteria, suggesting that lytic MACPF proteins may share a common mode of pore formation. By analogy to well-characterised members of the CDC family, such as pneumolysin (PLY) and streptolysin O, it appears the mechanism consists of initial membrane binding through a membrane proximal immunoglobulin domain. Upon further conformational changes, the monomers coalesce into a giant β-barrel-linked channel which perforates the target cell membrane. The detailed mechanism of this process has subsequently been confirmed both through mutagenesis studies and determination of the crystal structure of monomeric murine perforin (2.75 Å).[13,15]

Perforin is encoded on a single-copy gene in both mice and humans,[5] and while there is a level of redundancy to many of the granule components, perforin is indispensible for protective

Abbreviations: NK, natural killer; CTL, cytotoxic T lymphocyte; MACPF, membrane-attack complex/perforin; CDC, cholesterol-dependent cytolysin; PLY, pneumolysin; EGF, epidermal growth factor; FLH, familial hemophagocytic lymphohistiocytosis; SAR, structure–activity relationship; SEM, standard error of the mean.

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immunosurveillance. Perforin knock-out mice demonstrate the effect of deficiency in vivo through increased susceptibility and failure to clear many viruses and other intracellular pathogens, as well as the development of highly aggressive disseminated B cell lymphoma in the majority of animals over the age of 12 months.[16] In humans, complete loss of perforin function results in familial hemophagocytic lymphohistiocytosis (FLH) syndrome which is characterised by severe anemia and hepatosplenomegaly, fever, and thrombocytopenia.[17–20] Partial loss, depending upon the level of residual activity, is still strongly associated with FLH (often late onset) as well as a range of hematological malignancies, particularly in adolescence.[21]

CTL and NK cells have also been implicated in several autoimmune diseases (e.g., insulin-dependent diabetes),[4,22,23] therapy-induced conditions (e.g., allograft rejection, graft-versus-host disease),[24–26] and potentially fatal complications of malarial and viral infections (e.g., cerebral malaria, post viral myocarditis).[10] Current accepted immunosuppressive treatments are associated with a wide range of side-effects,[27,28] many of which arise from the non-specificity of therapies where multiple molecular targets are affected indiscriminately. Given the pivotal role performed by perforin in CTL and NK cell induced lysis, a small molecule inhibitor of perforin is of potential interest as a new class of highly specific immunosuppressive agents. In our initial work in this area, we have already explored two series of compounds, the dihydrofuro [3,4-*c*]pyridinones[29] and the 1-amino-2,4-dicyanopyrido[1,2-*a*] benzimidazoles,[30] both of which showed appreciable perforin-inhibitory activity. However we continue to seek improved potency and drug-like properties, and thus report here our current work ona series of aryl-substituted isobenzofuran-1(3*H*)-ones as small-molecule inhibitors of perforin-induced lysis.

2. Results and discussion

2.1. Chemistry

A high throughput screen[31] of approximately 100,000 compounds, sourced from commercial libraries, was carried out using a 384-well plate format. Compounds were screened (at 20 μM) by incubation with sheep red blood cells, which in the absence of an inhibitor are lysed by perforin, generating turbidity that can be measured by a change in absorbance at 650 nm. This resulted in the identification of a small number of hits which showed reproducible perforin-inhibitory activity for recombinant mouse and human perforin. These hits were then also screened for their ability to inhibit the perforin-induced lysis of ^{51}Cr-labelled Jurkat lymphoma cells and IC_{50}s calculated. Compared to the original first-in-class dihydrofuro[3,4-*c*]pyridinone perforin inhibitors,[29] as exemplified by compound **1** (IC_{50} = 0.97 μM), the new lead compound **2** showed moderate, but reproducible activity (IC_{50} = 6.20 μM; Fig 1).

Compound **2** can be readily disconnected into three subunits; a 2-thioxoimidazolidin-4-one (A), a furan (B) and a benzofuranone (C), a structure which lends itself to independent variation of each subunit and subsequent analysis of the resulting structure–activity relationships (SAR). In this study we explore the effect of varying the 2-thioxoimidazolidin-4-one A-subunit and the furan B-subunit on a fixed benzofuranone scaffold, on the ability of the resulting compounds to inhibit perforin lytic activity.

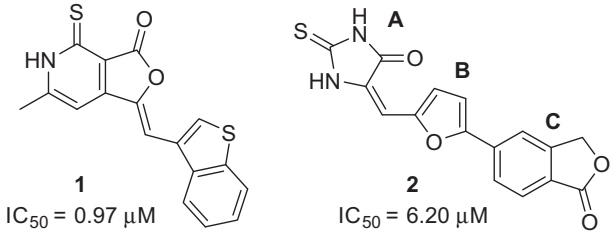


Figure 1. Original and new perforin inhibitor leads.

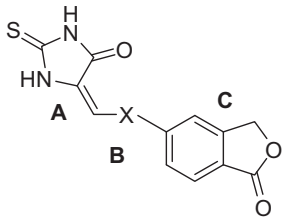
The compounds of Table 1 were prepared through a key Suzuki coupling with a variety of protected aldehydes (**3**–**21**) and boronate **22** (Scheme 1), to give intermediates **23–41**. The boronate **22** is easily prepared in gram quantities from 4-bromo-2-methylbenzoic acid[32,33] and was employed in the synthesis of compounds in both Tables 1 and 2. Many of the required aldehydes were commercially available and the 5- and 6-bromobenzo[*b*]thiophene-2-carboxaldehydes were prepared according to published procedures.[34,35] Deprotection of the Suzuki products **23**–**41** under acidic conditions gave a range of heterocyclic aldehydes **42**–**60** which were reacted with 2-thiohydantoin to give the target compounds **2**, **94**–**100**, **103**–**105**, **109**–**116**.

In order to expand our investigation into a range of heterocyclic B-subunits based on non-commercially available substrates, a selection of key substituted aldehydes **64**, **70**, **72**, **80**, **84** were prepared as described in Schemes 2–4. Pyrrole **64** was prepared by Suzuki reaction of BOC-protected pyrrole boronic acid **61** with 5-bromoisobenzofuran-1(3*H*)-one (**62**) (Scheme 2). This afforded intermediate **63** which was formylated under Vilsmeier conditions (with concomitant deprotection of the carbamate) to give the target aldehyde **64**. Oxazole- and isoxazole-5-carboxaldehydes **70** and **72** were both prepared from 1-oxo-1,3-dihydroisobenzofuran-5-carboxylic acid **65** (Scheme 3). In the case of the isoxazole, carboxylic acid **65** was reduced to the benzyl alcohol **66** by activation with CDI, followed by reduction with $NaBH_4$. Oxidation with PCC on silica gel gave the aldehyde **67** which was converted to the corresponding oxime **68** by reaction with hydroxylamine hydrochloride. The oxime **68** was ring-closed through chlorination with *N*-chlorosuccinimide and condensation with propargyl alcohol,[36] giving hydroxymethyl isoxazole **69**. A second oxidation step with PCC/silica gave the desired aldehyde **70**. For the oxazole, the carboxylic acid **65** was converted to the propargylic amide **71** by activation with pentafluorophenyl trifluoroacetate and reaction with propargylamine. Palladium-catalysed ring-closure to the oxazole using $PdCl_2(Bn)_2$ in the presence of $CuCl_2$ and under an atmosphere of oxygen[37] gave aldehyde **72** in low (17%) yield. The isomeric isothiazolecarboxaldehydes **80** and **84** were prepared from 1-oxo-1,3-dihydroisobenzofuran-5-carboxamide **75** (Scheme 4). The carboxamide **75** was reacted with chlorocarbonylsulfenyl chloride by heating in dioxane, giving 1,3,4-oxathiazol-2-one **76**, which in turn was condensed with ethyl propiolate and decarboxylated[38] to afford a mixture of isomeric ethyl isothiazolecarboxylates (**77**, **81**) which were separated by chromatography. The esters **77** and **81** were hydrolysed under basic conditions to the acids **78** and **82**, then reduced to the corresponding isoxazole alcohols **79** and **83**. Finally, oxidation with PCC/silica gave the desired aldehydes **80** and **84**.

With the target aldehydes **64**, **70**, **72**, **80**, **84** in hand, the corresponding final products **101**, **102**, **106**, **107**, **108** were prepared by reaction with 2-thiohydantoin in the presence of β-alanine and refluxing AcOH. A single acyclic analogue (**117**) was also prepared (Scheme 3); EDCI/HOBt-mediated coupling of the carboxylic acid **65** with 3-amino-1-propanol gave alcohol **73** which was oxidised to the aldehyde **74** with Dess–Martin periodinane. Reaction with 2-thiohydantoin as above gave the alkylamido-linked compound **117**.

The compounds of Table 2 were synthesized by Suzuki reaction of 2-bromothiophenes **4** and **85**–**87** with key boronate **22** (Scheme 5). This gave final products **118**, **119**, **120**, **121** (**121** from methylation of **120** with TMS-diazomethane) directly, and deprotection of the cyclic acetal **24** gave key intermediate aldehyde **43**, from which

Table 1
Inhibitory activities of isobenzofuran-1(3*H*)-ones with various B-subunits



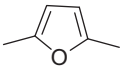
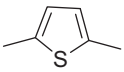
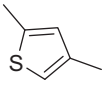
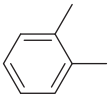
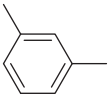
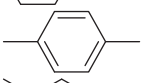
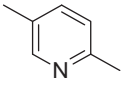
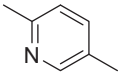
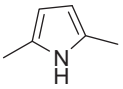
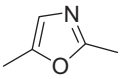
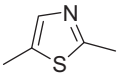
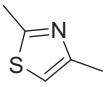
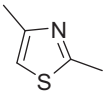
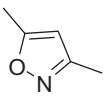
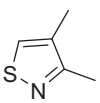
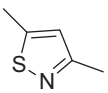
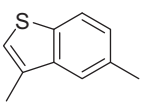
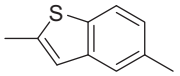
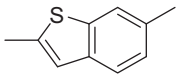
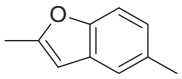
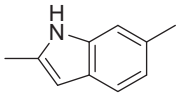
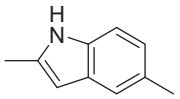
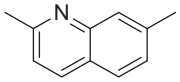
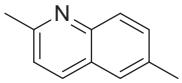
Number	X[a]	Jurkat IC_{50}[b] (μM)
2		6.20
94		0.78
95		1.05
96		>20
97		2.11
98		1.80
99		2.00
100		0.37
101		2.45
102		7.28
103		1.72
104		1.41
105		0.34
106		1.30
107		2.54
108		1.44
109		2.27

Table 1 (*continued*)

Number	X[a]	Jurkat IC_{50}[b] (μM)
110		0.49
111		0.36
112		0.51
113		0.36
114		0.82
115		1.72
116		0.47
117	CH_2CH_2NHCO	>20

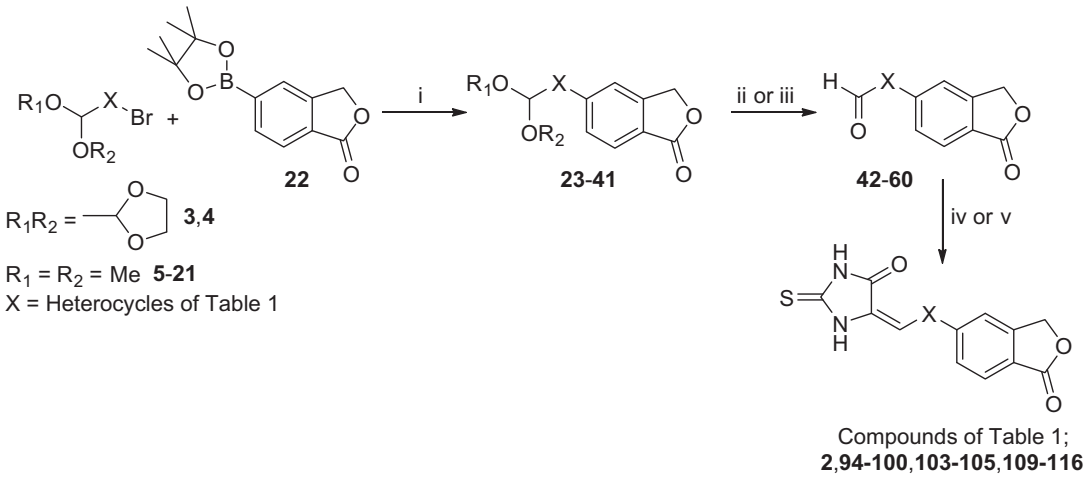
[a] Structure of the B-subunit.
[b] Testing was carried out over a range of doses, with the IC_{50} being equal to the concentration at which 50% inhibition of the lysis of Jurkat cells by perforin was observed, as measured by ^{51}Cr release. Values are the average of at least three independent IC_{50} determinations.

the remaining compounds of Table 2 (**122**–**134**) were prepared. Compounds **122**–**128** were prepared by simply heating aldehyde **43** with the appropriate heterocycle in the presence of β-alanine and refluxing AcOH. The oxazole **129** was obtained by heating **43** with tosylmethyl isocyanate and K_2CO_3 in MeOH (Scheme 5), and succinimide derivative **130** was isolated after reaction between **43** and triphenylphosphoranylidenesuccinimide[39] **89** (Scheme 6). Scheme 7 describes the preparation of the remaining targets, a series of five- and six-membered lactam derivatives (**131**–**134**). In the first instance, the benzyl-protected diethyl (2-oxopyrrolidin-3-yl)phosphonate **90** was prepared and reacted with **43** using KHMDS and 18-crown-6 at low temperature.[40] This gave compound **131** from which the benzyl protecting group was unable to be removed. These two steps were then repeated with the 4-methoxybenzyl-protected compound **91**, to give **132** which was successfully deprotected to the unsubstituted lactam **133** using TFA/anisole. Finally, this sequence was repeated to give the protected six-membered lactam **93** which was also successfully deprotected, affording **134**.

2.2. Structure–activity relationships

2.2.1. Effect of replacing the central furan ring (Table 1)

Lead compound **2** was employed as the starting point for the design and synthesis of a new series of aryl-substituted isobenzofuran-1(3*H*)-ones. All final products were then screened for their ability to inhibit the perforin-induced lysis of ^{51}Cr-labelled Jurkat lymphoma cells (for further details see Section 4). Our initial strategy was to retain the 2-thioxoimidazolidin-4-one A- and isobenzofuranone C-subunits, while exchanging the central furan ring for a variety of aromatic B-subunits. We first replaced the 2,5-furan of lead **2** with a 2,5-thiophene (**94**). This modification resulted in a very



Scheme 1. Reagents and conditions: (i) 2 M Na_2CO_3, toluene/EtOH, $Pd(dppf)Cl_2$, reflux, 2 h; (ii) 1 M HCl, acetone, rt; (iii) *p*-TsOH, water, acetone, 50 °C; (iv) 2-thiohydantoin, piperidine, EtOH, reflux, 15 h; (v) 2-thiohydantoin, β-alanine, AcOH, reflux, 15 h.

encouraging eightfold improvement in activity; IC_{50} = 0.78 μM for the thiophene, compared to 6.20 μM for the furan. The isomeric 2,4-thiophene (**95**) was also significantly more potent than the furan **2**, suggesting some level of flexibility in the angle of connection between the A- and C-subunits. This was further investigated via the synthesis of the three benzene-linked compounds **96**–**98**. It was established that the 1,4-linkage was optimal (**98**), with an IC_{50} = 1.80 μM. The 1,3- (**97**) and 1,2- (**96**) linked compounds were progressively less potent (IC_{50} = 2.11 and >20 μM, respectively). The two pyridyl-linked isomers **99** and **100** showed a similar trend, however in this case the position of the pyridine nitrogen also appears important, with the 2,5-linked compound **100** (0.37 μM) significantly more potent than its 3,6 counterpart **99** (2.00 μM).

While 2,5-pyrrole **101** showed only moderate activity (IC_{50} = 2.45 μM), the improvement in activity observed in the transition from furan **2** to thiophene **94** is replicated in the corresponding oxazole/thiazole pair **102** and **103** (IC_{50}s = 7.28 and 1.72 μM). 4-Thiazol-2-yl compound **104** also showed good activity (1.41 μM), but the outstanding isomer of the set was the 2-thiazol-4-yl analog **105** with an IC_{50} = 0.34 μM. Isoxazole **106** and isothiazoles **107** and **108** also all showed appreciable activity (1.30–2.54 μM).

Bicyclic B-units were then explored, with the benzothiophene series **109**–**111** showing a similar dependence on the angle of connection to the benzene series; the 2,5- and 2,6-isomers **110** and **111** (0.49 and 0.36 μM) being significantly more potent than the 3,5-isomer **109** (2.27 μM). Interestingly, unlike the furan/thiophene pair of **2** and **94**, the 2,5-benzofuran **112** and 2,5-benzothiophene **110** showed virtually identical activity (0.51 and 0.49 μM, respectively). Indoles **113**, **114** and quinoline **116** all showed sub-micromolar activity (0.36–0.82 μM), with quinoline **115** a little less potent at 1.72 μM. Finally, a single acylic compound **117** was prepared; it's inactivity confirming that an aromatic B-subunit is required, presumably by providing the additional rigidity needed for an active conformation.

2.2.2. Exploring the influence of the thioxoimidazolidinone (Table 2)

Having established a small group of preferred B-subunits, the influence of A-subunit was then explored. For this study, the B- and C-subunits were fixed as thiophene and isobenzofuran-1(3*H*)-one, respectively. While thiophene **94** was not the most potent compound of Table 1, it was selected based on a range of factors including inhibitory activity, synthetic accessibility, cost of goods and overall molecular weight of the final compound. Examples **118**, **119**, **120** and **121** (no activity) suggest a requirement for a ring as the A-subunit, the only exception being the aldehyde **43** which showed moderate activity (IC_{50} = 6.31 μM). Replacement of the 2-thioxoimidazolidin-4-one of **94** with a variety of related five-membered heterocycles (**122**, **124**, **128**) is tolerated (IC_{50}s = 1.12–2.75 μM), but all examples show less activity. It is unclear why the 2-thioxothiazolidin-4-one **123** is so much poorer than the other examples. Expansion of the thioxoimidazolidinone ring to a six-membered ring is permissible, as illustrated by compounds **125** and **126** which have comparable activity to **94**, but methylation of the ring nitrogens (**127**) results in significant loss of activity. Oxazole **129** also shows moderate activity.

Systematic deletion of NH and carbonyl groups from compound **122** (IC_{50} = 1.19 μM) to give the pyrrolidine-2,5-dione **130** and pyrrolidine-2-one **133** results in significant loss of potency (IC_{50}s = 6.97 and 6.81 μM, respectively) which is compounded by expansion of the ring to the six-membered analog **134** (IC_{50} = 15.2 μM). Compounds **131** and **132** also reinforce the need for the amido nitrogens to remain unsubstituted.

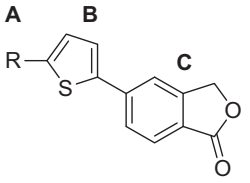
Another factor which contributes to the perforin-inhibitory activity of aryl-substituted isobenzofuran-1(3*H*)-ones is the presence of a double bond fused to the A-unit, resulting in a mixture of *E*- and *Z*-isomers. Reduction of this double bond in an active compound, to give its saturated analogue, results in complete loss of activity (data not shown). Analytical HPLC and ^{1}H NMR studies show gradual conversion of the predominant *Z*-isomer to the corresponding *E*-isomer in solution, ruling out any possibility of separation, and all compounds prepared have consequently been synthesised as a mixture of *E*- and *Z*-isomers (see Supplementary data for characterisation of isomers and stability studies).

2.2.3. Further analysis of compounds with perforin-inhibitory activity

Lead compound **2** was additionally tested for its ability to inhibit the lytic function of the pneumococcal toxin pneumolysin (PLY), a cholesterol-dependent pore-forming protein. PLY, like perforin, is released as a soluble monomer which assembles into oligomeric pores at the target cell membrane. Perforin lysis of ^{51}Cr labelled Jurkat cells is completely inhibited by **2** at a concentration of 20 μM. By substituting PLY for perforin under otherwise identical conditions, lysis was not inhibited by **2**, showing that it specifically inhibits perforin, but not the mechanistically-related PLY.

Those compounds which showed the ability to inhibit lysis of labelled Jurkat cells by purified perforin were then also evaluated

Table 2
Inhibitory activities of isobenzofuran-1(3*H*)-ones with various A-subunits



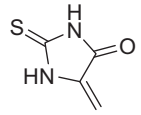
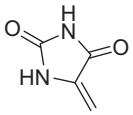
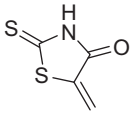
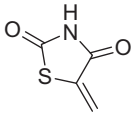
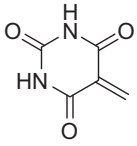
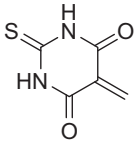
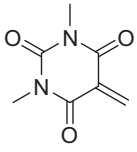
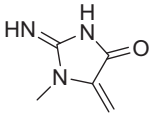
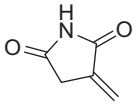
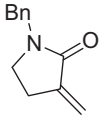
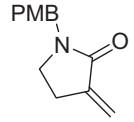
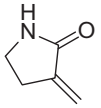
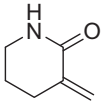
Number	R[a]	Jurkat IC_{50}[b] (μM)
118	H	>20
119	Acetyl	>20
43	CHO	6.31
120	CH_2COOH	>20
121	CH_2COOMe	>20
94		0.78
122		1.19
123		>10
124		1.12
125		0.80
126		0.40
127		3.96
128		2.75
129		3.18
130		6.97
131		>20

Table 2 (*continued*)

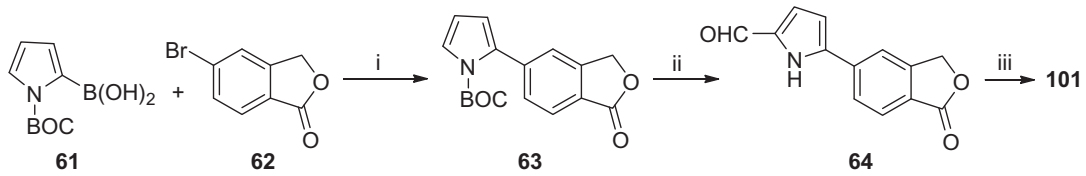
Number	R[a]	Jurkat IC_{50}[b] (μM)
132		>20
133		6.81
134		15.2

[a] Structure of the A-subunit.
[b] Testing was carried out over a range of doses, with the IC_{50} being equal to the concentration at which 50% inhibition of the lysis of Jurkat cells by perforin was observed, as measured by ^{51}Cr release. Values are the average of at least three independent IC_{50} determinations.

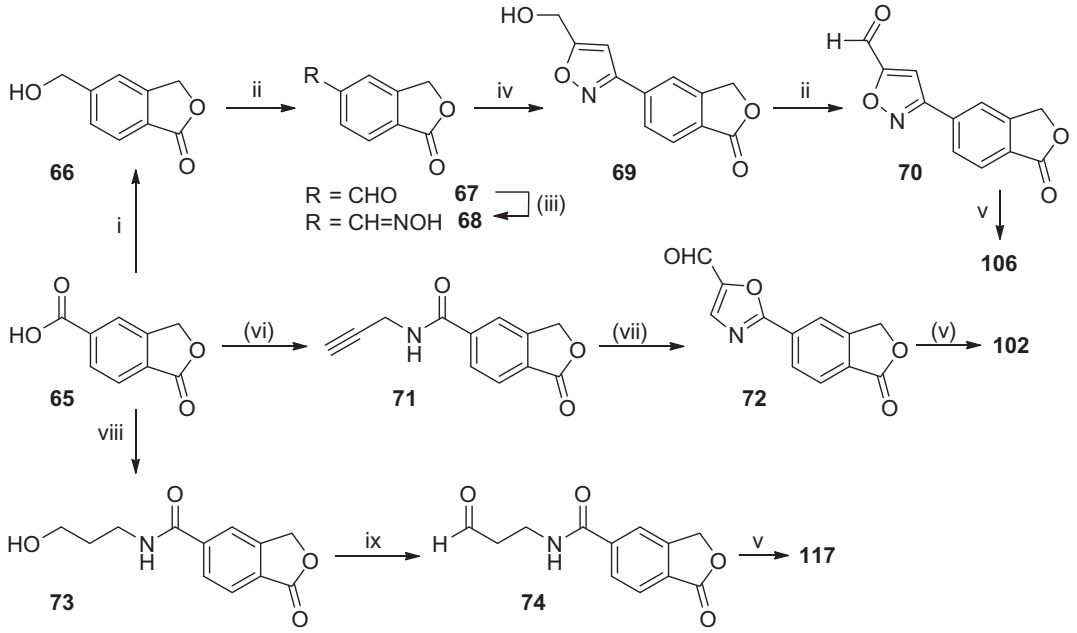
for their inhibition of perforin delivered by an intact NK cell line. The inhibitor (20 μM final concentration) and medium were co-incubated with KHYG-1 NK cells for 30 min at room temperature, ^{51}Cr-labelled K562 leukemia target cells were then added, and cell lysis evaluated after 4 h incubation at 37 °C by measuring ^{51}Cr release. This assay can be regarded as a more stringent test of activity than measurement of isolated perforin-mediated Jurkat cell lysis, because the perforin is delivered by a whole cell and is thus more representative of the situation in vivo. To confirm that the inhibitory activity exhibited by the compounds against KHYG-1 NK cells was due to blocking the action of perforin and not non-specific toxicity against the effector cell, the viability of the NK cells in the presence of inhibitor was also measured 24 h later. Viable and dead cells were counted and percent viability calculated based on total cell count (for further details see Section 4). The key role played by CTLs and NK cells in the overall immunological response means that it is essential that any pharmacological intervention allows rapid recovery of these cytotoxic effector cells, thus for the purposes of this study, compounds are classified as toxic if NK cell viability falls below 70%.

All compounds tested showed at least some suppression of NK cell killing of the K562 targets, ranging from 11% to 100% inhibition (Table 3), however most compounds also show an effect on the viability of the KHYG-1 NK cells (10–68% viability), suggesting that toxicity does make at least some contribution to the observed level of inhibition for these examples. Of particular note in this regard are indoles **113** and **114** which showed excellent activity against isolated perforin (IC_{50} = 0.36 and 0.82 μM), but clearly the outstanding activity observed with whole NK cells (100% and 91%, respectively) is significantly due to toxicity (10% and 23% viability). Compared to dihydrofuro[3,4-*c*]pyridinone **1** (41% inhibition of perforin-induced target cell killing), two compounds in the current series; thiophene-linked **94** andimidazolidine-2,4-dione **122**, exhibited improved effectiveness in this assay (53% and 54% inhibition, respectively) whilst demonstrating minimal KHYG-1 NK cell toxicity (both around 90% viability).

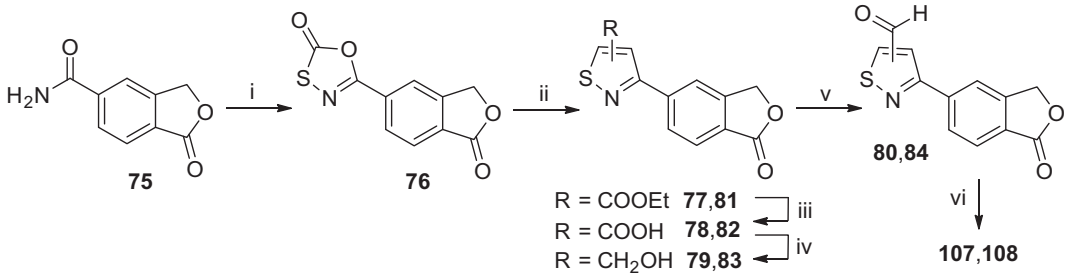
The most significant drawback of the original series of dihydrofuro[3,4-*c*]pyridinones (e.g. **1**) was that cellular activity was adversely affected by increasing concentrations of serum. The activity of **1** was reduced from 41% inhibition with no serum



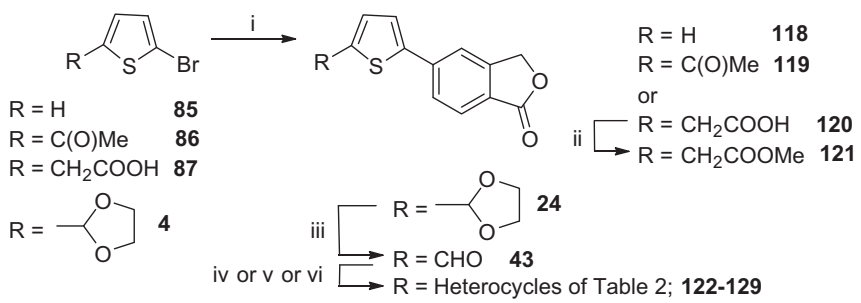
Scheme 2. Reagents and conditions: (i) 2 M Na_2CO_3, toluene/EtOH, Pd(dppf)Cl_2, reflux, 2 h; (ii) $POCl_3$, DMF, CH_2Cl_2, 0 °C to rt; (iii) 2-thiohydantoin, β-alanine, AcOH, reflux, 15 h.



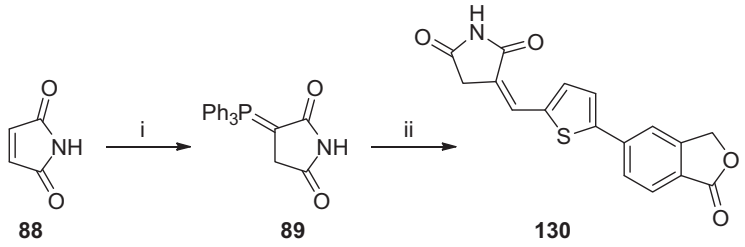
Scheme 3. Reagents and conditions: (i) (a) CDI, THF, rt; (b) $NaBH_4$, H_2O; (ii) PCC/silica, CH_2Cl_2; (iii) $NH_2OH{\cdot}HCl$, pyridine, MeOH, 60 °C, 1.5 h; (iv) (a) *N*-chlorosuccinimide, pyridine, THF, 60 °C; (b) propargyl alcohol, 50 °C, 2 h; (v) 2-thiohydantoin, β-alanine, AcOH, reflux, 15 h; (vi) (a) PFP-TFA, pyridine, rt; (b) propargylamine, THF, rt; (vii) Pd$(Bn)_2Cl_2$, O_2, $CuCl_2$, DMF, 100 °C; (viii) EDCI, HOBt, TEA, 3-amino-1-propanol, DMF, rt, 15 h; (ix) Dess–Martin periodinane, THF/DMF, rt, 3 h.



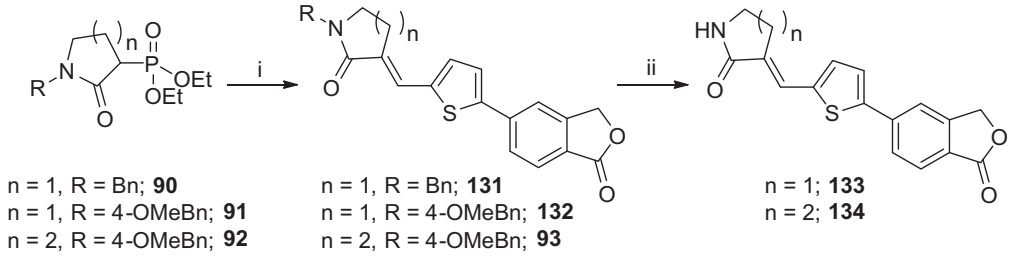
Scheme 4. Reagents and conditions: (i) chlorocarbonylsufenyl chloride, dioxane, 100 °C; (ii) ethyl propiolate, 1,2-dichlorobenzene, 150 °C, 5 h; (iii) (a) 2 M NaOH, EtOH, 50 °C; (b) TFA/CH_2Cl_2, rt; (iv) (a) CDI, THF, rt; (b) $NaBH_4$, H_2O; (v) PCC/silica, CH_2Cl_2; (vi) 2-thiohydantoin, β-alanine, AcOH, reflux, 15 h.



Scheme 5. Reagents and conditions: (i) **22**, 2 M Na_2CO_3, toluene/EtOH, Pd(dppf)Cl_2, reflux, 2 h; (ii) TMS-diazomethane, Et_2O/MeOH; (iii) 1 M HCl, acetone, rt; (iv) heterocycle (*R*) from Table 2, piperidine, EtOH, reflux, 15 h; (v) heterocycle (*R*) from Table 2, β-alanine, AcOH, reflux, 15 h; (vi) tosylmethyl isocyanide, K_2CO_3, MeOH, reflux, 1 h.



Scheme 6. Reagents and conditions: (i) Ph_3P, AcOH, 110 °C, 30 min; (ii) **43**, MeOH, reflux, 1 h.



Scheme 7. Reagents and conditions: (i) **43**, KHMDS, 18-crown-6, THF, −78 °C to rt; (ii) TFA, anisole, reflux, 15 h.

Table 3
Capacity of selected compounds to inhibit perforin delivered by KHYG-1 NK cells

Number	Jurkat IC_{50} (μM)	KHYG-1 inhibition (% at 20 μM)		KHYG-1 viability[c] (%)
		No serum[a]	10% serum[b]	
1	0.97	41 ± 3.6	10.3 ± 5.9	77 ± 12.0
2	6.20	74 ± 8.2	23 ± 6.7	66 ± 7.4
94	0.78	53 ± 2.6	38.9 ± 3.5	88 ± 3.0
95	1.05	49 ± 7.5	42 ± 10.5	55 ± 11.0
98	1.80	28 ± 4.3	30 ± 9.8	59 ± 16.0
100	0.37	37 ± 11.6	20 ± 6.6	92 ± 5.3
103	1.72	45 ± 6.0	27 ± 7.2	68 ± 12.0
104	1.41	30 ± 5.0	13 ± 7.7	91 ± 2.3
105	0.34	11 ± 4.8	2 ± 1.0	25 ± 10.0
106	1.30	41 ± 3.9	23 ± 8.6	63 ± 9.7
108	1.44	45 ± 8.3	26 ± 5.4	57 ± 11.0
110	0.49	51 ± 4.5	17 ± 4.8	27 ± 2.9
111	0.36	46 ± 8.3	25 ± 4.1	19.7 ± 11.0
112	0.51	61 ± 5.2	35 ± 8.7	29.9 ± 3.0
113	0.36	100 ± 2.0	100 ± 8.0	10 ± 2.0
114	0.82	91 ± 8.2	83 ± 8.6	23.2 ± 4.4
115	1.72	52 ± 5.0	38 ± 5.7	59 ± 9.0
116	0.47	60 ± 6.0	35 ± 7.7	48.5 ± 2.4
122	1.19	54 ± 5.0	64 ± 8.9	91.5 ± 4.6
124	1.12	36 ± 10.0	12 ± 10.0	19 ± 6.5
125	0.80	52 ± 4.4	10 ± 3.0	77.6 ± 8.2
126	0.40	91 ± 6.6	73 ± 11.0	38 ± 13.0

[a] Inhibition by compound (20 μM) of the perforin-induced lysis of K562 target cells when co-incubated with KHYG-1 human NK cells (see Section 4). Percent inhibition calculated compared to untreated control.
[b] As for a, but in the presence of 10% mouse serum.
[c] Viability of KHYG-1 NK cells after 24 h by Trypan blue exclusion assay (see Section 4). All results are the average of at least three separate determinations ± SEM.

(20 μM concentration), to 10% in the presence of 10% mouse serum, severely limiting further development of the series. While this is also evident for many of the compounds in the current series, of the two most active compounds, **94** shows only limited loss of activity, while **122** appears to demonstrate a small increase in activity (from 54% inhibition in the absence of serum, to 64% in its presence).

3. Conclusions

Starting with a high throughput screening lead of moderate potency (**2**), a novel series of aryl-substituted isobenzofuran-1(3*H*)-ones have been developed as potent inhibitors of the cytolytic effects of perforin in cells. Molecules with this function are of potential interest as a new class of highly specific immunosuppressive agents for the treatment of autoimmune diseases and therapy-induced conditions characterized by undesired perforin secretion.

The SAR around this class of compounds was explored by independent variation of the 2-thioxoimidazolidin-4-one A- and furan B-subunits on an isobenzofuranone scaffold. The particular pattern of hydrogen bond donors and acceptors present in the 2-thioxoimidazolidin-4-one appears optimal for inhibition of isolated perforin protein, as none of the closely related five-membered replacements were as potent and all other deletions or substitutions resulted only in loss of activity. While the six-membered heterocyclic ring analogs (**125**, **126**) appeared at least as potent

against isolated perforin, extreme insolubility precluded further development. The area where the greatest gains in potency were found was in the linking B-subunit. Simply replacing 2,5-furan **2** with 2,5-thiophene **94** resulted in an eightfold increase in activity. Several compounds containing bicyclic B-subunits (**105**, **110**–**113**) were also potent inhibitors of perforin (IC_{50}s = 0.34–0.51 μM), showing between 12- and 18-fold more activity than the original lead **2**.

Although a wide range of compounds showed excellent activity toward isolated perforin protein, unfortunately many also proved toxic to the effector cell when whole NK cells were employed to deliver perforin. The overall best compound in this study, therefore, was imidazolidine-2,4-dione **122** which showed good inhibitory activity against isolated perforin (IC_{50} = 1.19 μM) and demonstrated excellent suppression of KHYG-1 NK cell mediated lysis in the presence of serum (64%) without toxicity toward the effector NK cells (>90% survival).

While it is noted that moieties similar to the 2-thioxoimidazolidin-4-one and imidazolidine-2,4-dione (thiazolidinediones, rhodanines) are widely reported in the literature and have been described as 'frequent (or promiscuous) hitters',[41] there remain many examples of such substructures being present in clinical drugs.[42] As observed above, lead compound **2** is a specific inhibitor of perforin, and at least in the case of PLY, is not a more general inhibitor of a mechanistically related pore-forming protein. In the present study, the 2-thioxoimidazolidin-4-one subunit is present in the majority of compounds synthesised, yet a wide range of activities is still observed against isolated perforin protein (IC_{50}s from 0.34 to >20 μM) as well as cell killing induced by intact NK cells (KHYG-1 inhibition for non-toxic active compounds tested range = 10–64%). These data demonstrate that the compounds possess the capability to specifically target perforin-dependent CTL and NK cell induced lysis.

Our original publication[29] described the first class of small molecules reported to inhibit the cytolytic effects of perforin in cells, but the SAR was relatively flat (<3-fold improvement over the original lead) and further development was precluded by inactivity in the presence of serum. The current series shows significant improvement in activity over original hit **2**, but more importantly, activity is retained in the presence of serum. Further work to elucidate the detailed mechanism of inhibition is on-going, including attempts to obtain a co-crystal structure of drug bound in the target protein. The results from this study now underpin our current work to further refine the aryl-substituted isobenzofuran-1(3*H*)-one series in order to identify a suitable in vivo candidate.

4. Experimental section

4.1. Chemistry

Analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined using an Electrothermal Model 9200 and are as read. NMR spectra were measured on a Bruker Advance 400 MHz spectrometer and referenced to Me_4Si. Mass spectra were recorded either on a Varian VG 7070 spectrometer at nominal 5000 resolution or a Finnigan MAT 900Q spectrometer. All final compound purities were determined to be >95% by HPLC on an Alltech Alltima C18 column (3.2 × 150 mm, 5 μm) eluting with 5–80% MeCN/45 mM NH_4HCO_3.

4.1.1. General procedure A: 5-(5-(1,3-dioxolan-2-yl)furan-2-yl) isobenzofuran-1(3*H*)-one (23)

5-Bromo-2-furaldehyde was protected as the cyclic acetal **3** according to a literature procedure.[43] The cyclic acetal (666 mg, 3.04 mmol) was dissolved in toluene (27 mL), to which was added a suspension of 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)isobenzofuran-1(3*H*)-one **22** (527 mg, 2.03 mmol) in EtOH (10 mL). This boronate ester was prepared in turn from 5-bromoisobenzofuran-1(3*H*)-one[33] **62** according to a literature procedure.[34] A solution of 2 M Na_2CO_3 (6.6 mL) and Pd(dppf)Cl_2 (90 mg, 0.10 mmol) were added and the entire mixture was heated at reflux under nitrogen for 2 h. Upon cooling, all solvents were removed under reduced pressure and the resulting residue was partitioned between water (50 mL) and CH_2Cl_2 (50 mL). Two further CH_2Cl_2 (50 mL) extractions were performed, then the combined organic fractions dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure to afford a residue which was purified by flash column chromatography on silica gel (10% EtOAc/hexanes as eluant). The title compound **23** was isolated as a beige solid (416 mg, 75%). ^{1}H NMR [$(CD_3)_2SO$] δ 7.98 (s, 1H), 7.92 (dd, *J* = 8.1, 1.2 Hz, 1H), 7.88 (d, *J* = 8.1 Hz, 1H), 7.21 (d, *J* = 3.5 Hz, 1H), 6.73 (d, *J* = 3.4 Hz, 1H), 5.98 (s, 1H), 5.44 (s, 2H), 4.05–4.14 (m, 2H), 3.94–4.03 (m, 2H).

4.1.2. General procedure B: 5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)furan-2-carbaldehyde (42)

Compound **23** (416 mg, 1.53 mmol) was dissolved in acetone (20 mL), to which was added 1 M HCl (4 mL). The resulting solution was stirred at rt for 3 h., at which point an off-white solid had precipitated. The mixture was diluted with water (100 mL) and extracted with CH_2Cl_2 (4 × 100 mL). The combined CH_2Cl_2 fractions were dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure to give a solid which was triturated with Et_2O to afford **42** as a beige solid upon filtration (292 mg, 84%); mp (Et_2O) 229–231 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 9.68 (s, 1H), 8.17 (br s, 1H), 8.09 (dt, *J* = 8.0, 0.7 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.71 (d, *J* = 3.8 Hz, 1H), 7.53 (d, *J* = 3.8 Hz, 1H), 5.48 (s, 2H). Anal. ($C_{13}H_8O_4$) H, C; +0.5.

4.1.3. General procedure C: (*E,Z*)-5-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)furan-2-yl)methylene)-2-thioxoimidazolidin-4-one (2)

Compound **42** (50 mg, 0.22 mmol) was suspended in EtOH (10 mL), to which was added 2-thiohydantoin (26 mg, 0.23 mmol) and piperidine (1 drop). This mixture was stirred at rt for 72 h. The title compound **2** was collected by filtration, directly from the reaction mixture, as a brown solid (57 mg, 80%); mp (EtOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.20 (br s, 1 H), 12.02 (br s, 1H), 8.18 (br s, 1H), 8.15 (d, *J* = 8.2 Hz, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.44 (d, *J* = 3.8 Hz, 1H), 7.38 (d, *J* = 3.8 Hz, 1H), 6.44 (s, 1H), 5.49 (s, 2H). LRMS (APCI$^+$) calcd for $C_{16}H_{11}N_2O_4S$ 327 (MH^+), found 327. Anal. ($C_{16}H_{10}N_2O_4S{\cdot}0.75H_2O$) C, H, N.

4.1.4. 5-(5-(1,3-Dioxolan-2-yl)thiophen-2-yl)isobenzofuran-1(3*H*)-one (24)

5-Bromo-2-thiophenecarboxaldehyde was protected as the cyclic acetal **4** according to a literature procedure[43] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (CH_2Cl_2 as eluant) gave **24** as a cream solid (56%). ^{1}H NMR [$(CD_3)_2SO$] δ 7.95 (br s, 1H), 7.84–7.90 (m, 2H), 7.63 (d, *J* = 3.8 Hz, 1H), 7.29 (d, *J* = 3.7 Hz, 1H), 6.08 (s, 1H), 5.43 (s, 2H), 4.01–4.11 (m, 2H), 3.92–4.00 (m, 2H). LRMS (APCI$^+$) calcd for $C_{15}H_{13}O_4S$ 289 (MH^+), found 289.

4.1.5. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophene-2-carbaldehyde (43)

Compound **24** was deprotected according to general procedure B. Trituration with Et_2O gave **43** as a beige solid (89%); mp (Et_2O) 224–226 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 9.96 (s, 1H), 8.12 (br s, 1H), 8.11 (d, *J* = 4.0 Hz, 1H), 8.02 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.93 (d, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 4.0 Hz, 1H), 5.47 (s, 2H). LRMS (APCI$^+$) calcd for $C_{13}H_9O_3S$ 245 (MH^+), found 245. Anal. ($C_{13}H_8O_3$) H, C; +0.5.

4.1.6. General procedure D: (*E,Z*)-5-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)-2-thioxoimidazolidin-4-one (94)

Compound **43** (50 mg, 0.21 mmol), 2-thiohydantoin (26 mg, 0.23 mmol) and β-alanine (20 mg, 0.23 mmol) were suspended in AcOH (5 mL) and the mixture heated at reflux for 15 h. Upon cooling, a yellow-brown solid crystallized out of solution and was collected by filtration. This material did not require further purification. The title compound **94** was isolated as an orange-brown solid after drying under vacuum (52 mg, 78%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.40 (br s, 1H), 12.02 (br s, 1H), 7.99 (br s, 1H), 7.93 (dd, J = 8.1, 1.4 Hz, 1H), 7.83–7.91 (m, 3H), 6.65 (s, 1H), 5.45 (s, 2H). Anal. ($C_{16}H_{10}N_2O_3S_2$) H, N, C; +0.5.

4.1.7. 5-(5-(Dimethoxymethyl)thiophen-3-yl)isobenzofuran-1(3*H*)-one (25)

4-Bromo-2-thiophenecarboxaldehyde was protected as the dimethyl acetal **5** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **25** as a white solid (44%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.11 (d, J = 1.6 Hz, 1H), 8.01 (d, J = 0.6 Hz, 1H), 7.94 (dd, J = 8.0, 1.4 Hz, 1H), 7.85 (d, J = 8.0 Hz, 1H), 7.62 (dd, J = 1.5, 0.8 Hz, 1H), 5.69 (s, 1H), 5.43 (s, 2H), 3.28 (s, 6H). LRMS (APCI$^+$) calcd for $C_{15}H_{15}O_4S$ 291 (MH^+), found 291.

4.1.8. General procedure E: 4-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophene-2-carbaldehyde (44)

Compound **25** (98 mg, 0.34 mmol) was dissolved in a mixture of acetone (6 mL) and water (2 mL), then toluenesulfonic acid (20 mg) added, and the mixture stirred at 50 °C for 3 h. After cooling, the reaction mixture was partitioned between satd $NaHCO_3$ solution (50 mL) and CH_2Cl_2 (50 mL). An additional extraction was carried out with CH_2Cl_2 (50 mL), then the combined organic extracts dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure to afford **44** which did not require further purification and was isolated as a white solid (87%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.00 (d, J = 1.3 Hz, 1H), 8.67 (t, J = 1.4 Hz, 1H), 8.59 (d, J = 2.0 Hz, 1H), 8.08 (d, J = 0.6 Hz, 1H), 8.01 (dd, J = 8.1, 1.4 Hz, 1H), 7.93 (d, J = 7.9 Hz, 1H), 5.48 (s, 2H).

4.1.9. (*E,Z*)-5-((4-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)-2-thioxoimidazolidin-4-one (95)

Reaction of **44** with 2-thiohydantoin according to general procedure D, followed by trituration with dioxane gave **95** as a yellow solid (80%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.39 (s, 1H), 12.21 (s, 1H), 8.30–8.35 (m, 2H), 8.07 (br d, J = 8.3 Hz, 2H), 7.94 (d, J = 8.1 Hz, 1H), 6.56 (s, 1H), 5.49 (s, 2H). LRMS (APCI$^-$) calcd for $C_{16}H_9N_2O_3S_2$ 341 (M−H), found 341. Anal. ($C_{16}H_{10}N_2O_3S_2{\cdot}0.25H_2O$) C, H, N.

4.1.10. 5-(2-(Dimethoxymethyl)phenyl)isobenzofuran-1(3*H*)-one (26)

2-Bromobenzaldehyde was protected as the dimethyl acetal **6** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **26** as a white solid (53%). ^{1}H NMR [$(CD_3)_2SO$] δ 7.92 (d, J = 7.8 Hz, 1H), 7.61–7.66 (m, 2H), 7.55 (dd, J = 7.9, 1.3 Hz, 1H), 7.44–7.51 (m, 2H), 7.27–7.33 (m, 1H), 5.47 (s, 2H), 5.14 (s, 1H), 3.28 (s, 3H), 3.16 (s, 3H). LRMS (APCI$^+$) calcd for $C_{17}H_{17}O_4$ 285 (MH^+), found 285.

4.1.11. 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzaldehyde (45)

Compound **26** was deprotected according to general procedure E to give **45** as a cream solid (93%). ^{1}H NMR [$(CD_3)_2SO$] δ 9.91 (s, 1H), 7.99 (dd, J = 7.8, 1.2 Hz, 1H), 7.95 (d, J = 7.9 Hz, 1H), 7.81 (td, J = 7.5, 1.4 Hz, 1H), 7.76 (d, J = 0.7 Hz, 1H), 7.63–7.70 (m, 2H), 7.55 (dd, J = 7.7, 0.8 Hz, 1H), 5.49 (s, 2H). LRMS (APCI$^+$) calcd for $C_{15}H_{11}O_3$ 239 (MH^+), found 239.

4.1.12. (*E,Z*)-5-(2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzylidene)-2-thioxoimidazolidin-4-one (96)

Reaction of **45** with 2-thiohydantoin according to general procedure D gave a solid which was isolated by filtration from the reaction mixture and did not require further purification. The desired product **96** was obtained as a yellow solid (95%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.25 (br s, 2H), 7.92 (d, J = 7.9 Hz, 1H), 7.81–7.87 (m, 1H), 7.68 (d, J = 0.5 Hz, 1H), 7.49–7.57 (m, 3H), 7.41–7.46 (m, 1H), 6.15 (s, 1H), 5.45 (s, 2H). Anal. ($C_{18}H_{12}N_2O_3S$) C, H, N.

4.1.13. 5-(3-(Dimethoxymethyl)phenyl)isobenzofuran-1(3*H*)-one (27)

3-Bromobenzaldehyde was protected as the dimethyl acetal **7** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **27** as a white solid (59%). ^{1}H NMR [$(CD_3)_2SO$] δ 7.97 (d, J = 0.5 Hz, 1H), 7.93 (d, J = 8.0 Hz, 1H), 7.87 (dd, J = 8.0, 1.4 Hz, 1H), 7.72–7.77 (m, 2H), 7.52–7.58 (m, 1H), 7.47 (d, J = 7.7 Hz, 1H), 5.48 (s, 1H), 5.47 (s, 2H), 3.30 (s, 6H). LRMS (APCI$^+$) calcd for $C_{17}H_{17}O_4$ 285 (MH^+), found 285.

4.1.14. 3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzaldehyde (46)

Compound **27** was deprotected according to general procedure E, giving **46** which did not require further purification and was isolated as a cream solid (86%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.13 (s, 1H), 8.30 (t, J = 1.6 Hz, 1H), 8.12 (dq, J = 7.7, 1.9, 1.1 Hz, 1H), 8.06 (t, J = 0.9 Hz, 1H), 8.00 (dt, J = 7.7, 1.3 Hz, 1H), 7.97 (d, J = 1.1 Hz, 2H), 7.77 (t, J = 7.7 Hz, 1H), 5.49 (s, 2H). LRMS (APCI$^+$) calcd for $C_{15}H_{11}O_3$ 239 (MH^+), found 239.

4.1.15. (*E,Z*)-5-(3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzylidene)-2-thioxoimidazolidin-4-one (97)

Reaction of **46** with 2-thiohydantoin according to general procedure D, followed by trituration with Et_2O gave **97** as a yellow solid (65%); mp (AcOH) 285–288 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.33 (br s, 2H), 8.04 (br s, 2H), 7.92–8.00 (m, 2H), 7.81 (br d, J = 7.8 Hz, 1H), 7.74 (br d, J = 8.0 Hz, 1H), 7.57 (t, J = 7.8 Hz, 1H), 6.58 (s, 1H), 5.50 (s, 2H). LRMS (APCI$^-$) calcd for $C_{18}H_{11}N_2O_3S$ 335 (M−H), found 335. Anal. ($C_{18}H_{12}N_2O_3S{\cdot}0.5H_2O$) C, H, N.

4.1.16. 5-(4-(Dimethoxymethyl)phenyl)isobenzofuran-1(3*H*)-one (28)

4-Bromobenzaldehyde was protected as the dimethyl acetal **8** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **28** as a white solid (51%). ^{1}H NMR [$(CD_3)_2SO$] δ 7.97 (d, J = 0.4 Hz, 1H), 7.93 (dd, J = 8.0, 0.5 Hz, 1H), 7.88 (dd, J = 8.0, 1.4 Hz, 1H), 7.78 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.2 Hz, 2H), 5.47 (s, 2H), 5.46 (s, 1H), 3.30 (s, 6H). LRMS (APCI$^+$) calcd for $C_{17}H_{17}O_4$ 285 (MH^+), found 285.

4.1.17. 4-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzaldehyde (47)

Compound **28** was deprotected according to general procedure E to give **47** as a white solid (87%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.10 (s, 1H), 8.03–8.08 (m, 3H), 7.95–8.02 (m, 4H), 5.49 (s, 2H). LRMS (APCI$^+$) calcd for $C_{15}H_{11}O_3$ 239 (MH^+), found 239.

4.1.18. (*E,Z*)-5-(4-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzylidene)-2-thioxoimidazolidin-4-one (98)

Reaction of **47** with 2-thiohydantoin according to general procedure D gave **98** as a yellow solid (89%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.29 (s, 2H), 8.03 (br s, 1H), 7.88–7.97 (m, 4H), 7.83 (d, J = 8.5 Hz, 2H), 6.54 (s, 1H), 5.48 (s, 2H). LRMS (APCI$^-$) calcd for $C_{18}H_{11}N_2O_3S$ 335 (M−H), found 335. Anal. ($C_{18}H_{12}N_2O_3S$) C, H, N.

4.1.19. 5-(6-(Dimethoxymethyl)pyridin-3-yl)isobenzofuran-1(3*H*)-one (29)

2-Bromopyridine-5-carboxaldehyde was protected as the dimethyl acetal **9** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (CH_2Cl_2, followed by 10% acetone/CH_2Cl_2 as eluant) gave **29** as a white solid (34%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.79 (s, 1H), 8.19 (s, 1H), 8.13 (d, J = 8.1 Hz, 1H), 8.01 (d, J = 8.0 Hz, 1H), 7.91 (dd, J = 8.2, 2.0 Hz, 1H), 7.82 (d, J = 8.2 Hz, 1H), 5.52 (s, 1H), 5.45 (s, 2H), 3.38 (s, 6H). LRMS (APCI$^+$) calcd for $C_{16}H_{16}NO_4$ 286 (MH^+), found 286.

4.1.20. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)picolinaldehyde (48)

Compound **29** was deprotected according to general procedure E to give **48** as a white solid (41%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.18 (s, 1H), 9.22 (dd, J = 2.1, 0.8 Hz, 1H), 8.48 (br s, 1H), 8.37–8.42 (m, 2H), 8.33 (d, J = 8.2 Hz, 1H), 8.00 (d, J = 8.2 Hz, 1H), 5.52 (s, 2H). LRMS (APCI$^+$) calcd for $C_{14}H_{10}NO_3$ 240 (MH^+), found 240.

4.1.21. (*E,Z*)-5-((6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)pyridine-3-yl)methylene (99)

Reaction of **48** with 2-thiohydantoin according to general procedure D gave **99** as a yellow solid (89%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.38 (br s, 2H), 9.00 (d, J = 2.2 Hz, 1H), 8.45 (s, 1H), 8.38 (d, J = 8.1 Hz, 2H), 8.17 (d, J = 8.4 Hz, 1H), 7.97 (d, J = 8.1 Hz, 1H), 6.57 (s, 1H), 5.50 (s, 2H). LRMS (APCI$^-$) calcd for $C_{17}H_{10}N_3O_3S$ 336 (M−H), found 336. Anal. ($C_{17}H_{11}N_3O_3S$) C, H, N.

4.1.22. 5-(6-(Dimethoxymethyl)pyridin-3-yl)isobenzofuran-1(3*H*)-one (30)

5-Bromopyridine-2-carboxaldehyde was protected as the dimethyl acetal **10** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (CH_2Cl_2, followed by 10% acetone/CH_2Cl_2 as eluant) gave **30** as a off-white solid (68%). ^{1}H NMR ($CDCl_3$) δ 8.87 (d, J = 1.9 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.97 (dd, J = 8.1, 2.4 Hz, 1H), 7.74 (d, J = 8.1 Hz, 1H), 7.66–7.72 (m, 2H), 5.45 (s, 1H), 5.40 (s, 2H), 3.45 (s, 6H). LRMS (APCI$^+$) calcd for $C_{16}H_{16}NO_4$ 286 (MH^+), found 286.

4.1.23. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)picolinaldehyde (49)

Compound **30** was deprotected according to general procedure E to give **49** as a off-white solid (88%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.06 (s, 1H), 9.23 (dd, J = 2.3, 0.7 Hz, 1H), 8.44 (ddd, J = 8.1, 2.3, 0.7 Hz, 1H), 8.16 (d, J = 0.7 Hz, 1H), 7.98–8.09 (m, 3H), 5.51 (s, 2H). LRMS (APCI$^+$) calcd for $C_{14}H_{10}NO_3$ 240 (MH^+), found 240.

4.1.24. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)pyridin-2-yl)methylene)-2-thioxoimidazolidin-4-one (100)

Reaction of **49** with 2-thiohydantoin according to general procedure D, gave **100** as a yellow-green solid (80%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.54 (br s, 1H), 11.52 (br s, 1H), 9.15 (d, J = 2.4 Hz, 1H), 8.29 (dd, J = 8.2, 2.5 Hz, 1H), 8.09 (s, 1H), 7.96–8.02 (m, 2H), 7.87 (d, J = 8.2 Hz, 1H), 6.69 (s, 1H), 5.50 (s, 2H). LRMS (APCI$^-$) calcd for $C_{17}H_{10}N_3O_3S$ 336 (M−H), found 336. Anal. ($C_{17}H_{11}N_3O_3S$) C, H, N.

4.1.25. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-pyrrole-2-carbaldehyde (64)

Compound **62** (1.01 g, 4.74 mmol) and 1-(*t*-butoxycarbonyl)pyrrole-2-boronic acid **61** were reacted according to general procedure A and partially purified by flash column chromatography on silica gel (10% EtOAc/hexanes as eluant). The crude product, *tert*-butyl 2-(1-oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-pyrrole-1-carboxylate **63**, was obtained as a pale yellow oil (704 mg, 50%) and used directly in the next step.

DMF (150 mg, 2.06 mmol) was added to a flask and cooled to 0 °C. $POCl_3$ (316 mg, 2.06 mmol) was added dropwise and the mixture stirred without cooling for 15 min. 1,2-Dichloroethane (1.0 mL) was added and the mixture cooled back to 0 °C. A solution of **63** (559 mg, 1.87 mmol) in dichloroethane (1.0 mL) was added dropwise and the resulting bright orange solution heated at reflux for 15 min. Upon cooling to room temperature, NaOAc (845 mg, 10.3 mmol) in water (3.7 mL) was added and heating at reflux continued for a further 15 min. The mixture was allowed to cool, then extracted with CH_2Cl_2 (4 × 50 mL) and the combined organic fractions washed with satd $NaHCO_3$ (100 mL) and dried (Na_2SO_4). Filtration and removal of the solvent under reduced pressure gave a crude product (with the BOC deprotected) which was purified by flash column chromatography on silica gel (5% acetone/CH_2Cl_2 as eluant), followed by trituration with Et_2O. The title compound **64** was isolated as a beige solid (95 mg, 22%); mp (Et_2O) 275–278 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.64 (s, 1H), 9.57 (s, 1H), 8.16 (s, 1H), 8.11 (dd, J = 1.8, 1.4 Hz, 1H), 7.87 (d, J = 8.1 Hz, 1H), 7.13 (dd, J = 3.9, 2.2 Hz, 1H), 6.99 (dd, J = 4.0, 2.4 Hz, 1H), 5.45 (s, 2H). LRMS (APCI$^+$) calcd for $C_{13}H_{10}NO_3$ 228 (MH^+), found 228. Anal. ($C_{13}H_9NO_3$) C, H, N.

4.1.26. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-pyrrol-2-yl)methylene)-2-thioxoimidazolidin-4-one (101)

Reaction of **64** with 2-thiohydantoin according to general procedure D gave **101** as a dark red solid (58%); mp (dioxane) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 13.01 (s, 1H), 12.51 (br s, 1H), 12.19 (br s, 1H), 7.91 (d, J = 8.0 Hz, 1H), 7.88 (br s, 1H), 7.85 (dd, J = 8.0, 1.3 Hz, 1H), 7.05 (br dd, J = 3.8, 2.5 Hz, 1H), 6.99 (br dd, J = 3.8, 2.0 Hz, 1H), 6.65 (s, 1H), 5.45 (s, 2H). Anal. ($C_{16}H_{11}N_3O_3S$·0.25AcOH) C, H, N.

4.1.27. 1-Oxo-*N*-(prop-2-ynyl)-1,3-dihydroisobenzofuran-5-carboxamide (71)

1-Oxo-1,3-dihydroisobenzofuran-5-carboxylic acid **65** (370 mg, 2.08 mmol) was dissolved in THF (15 mL). To this solution was added pyridine (1.64 g, 20.8 mmol), followed by pentafluorophenyltrifluoroacetate (2.91 g, 10.4 mmol) and the resulting mixture stirred at rt for 2 h. All solvent was removed under reduced pressure to give an oil which was dissolved in EtOAc (100 mL) and washed with 1 M HCl (2 × 50 mL), water (50 mL), satd $NaHCO_3$ (2 × 50 mL) and brine (50 mL). The organic layer was dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure to give a crude product which was purified by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) to give the intermediate pentafluorophenyl ester (666 mg, 93%) which was used immediately in the next step.

The above ester (666 mg, 1.94 mmol) was dissolved in THF (15 mL), to which was added propargylamine (160 mg, 2.90 mmol). This mixture was stirred at rt for 0.5 h, then all solvent removed under reduced pressure to yield a crude solid which was purified by flash column chromatography on silica gel (CH_2Cl_2 followed by 5% MeOH/CH_2Cl_2 as eluant). The title compound **71** was isolated as white needles (350 mg, 84%); mp (Et_2O) 242–245 °C dec. ^{1}H NMR

[$(CD_3)_2SO$] δ 9.19 (t, J = 5.5 Hz, 1H), 8.10 (s, 1H), 8.01 (dd, J = 8.0, 1.4 Hz, 1H), 7.94 (d, J = 7.8 Hz, 1H), 5.47 (s, 2H), 4.10 (dd, J = 5.5, 2.5 Hz, 2H), 3.14 (t, J = 2.5 Hz, 1H). LRMS ($APCI^+$) calcd for $C_{12}H_{10}NO_3$ 216 (MH^+), found 216. Anal. ($C_{12}H_9NO_3$) C, H, N.

4.1.28. 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)oxazole-5-carbaldehyde (72)

$PdCl_2(BnCN)_2$ (9 mg, 0.02 mmol) and $CuCl_2.2H_2O$ (8 mg, 0.05 mmol) in DMF were stirred at rt for 1 h. A solution of **71** (100 mg, 0.47 mmol) was added and the entire mixture stirred at 100 °C under an atmosphere of oxygen for 2 h. Upon cooling, the reaction mixture was partitioned between EtOAc (50 mL) and brine (50 mL). A precipitate formed which was collected by filtration. The EtOAc fraction was dried (Na_2SO_4) and the solvent removed under reduced pressure to give a solid which was combined with the above precipitate. The crude product was purified by flash column chromatography on silica gel (5% MeOH/CH_2Cl_2as eluant) to give **72** as a cream solid (18 mg, 17%). ^1H NMR [$(CD_3)_2SO$] δ 9.87 (s, 1H), 8.42 (s, 2H), 8.28 (dd, J = 8.0, 1.4 Hz, 1H), 8.05 (d, J = 8.1 Hz, 1H), 5.52 (s, 2H). HRMS (FAB^+) calcd for $C_{12}H_8NO_4$ 230.0453 (MH^+), found 230.0451.

4.1.29. (*E,Z*)-5-((2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)oxazol-5-yl)methylene)-2-thioxoimidazolidin-4-one (102)

Reaction of **72** with 2-thiohydantoin according to general procedure D gave **102** as a bright yellow solid (71%); mp (dioxane) >295 °C. ^1H NMR [$(CD_3)_2SO$] δ 12.54 (br s, 1H), 12.32 (br s, 1H), 8.45–8.51 (m, 2H), 8.06 (dd, J = 8.0, 0.4 Hz, 1H), 8.00 (d, J = 0.4 Hz, 1H), 6.54 (s, 1H), 5.54 (s, 2H). LRMS ($APCI^-$) calcd for $C_{15}H_8N_3O_4S$ 326 (M−H), found 326. Anal. ($C_{15}H_9N_3O_4S$) C, H, N.

4.1.30. 5-(5-(Dimethoxymethyl)thiazol-2-yl)isobenzofuran-1(3*H*)-one (31)

2-Bromo-1,3-thiazole-5-carboxaldehyde was protected as the dimethyl acetal **11** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **31** as a white solid (25%). ^1H NMR [$(CD_3)_2SO$] δ 8.25 (s, 1H), 8.15 (dt, J = 8.0, 0.6 Hz, 1H), 7.98 (d, J = 0.7 Hz, 1H), 7.95 (d, J = 8.0 Hz, 1H), 5.81 (d, J = 0.7 Hz, 1H), 5.48 (s, 2H), 3.35 (s, 6H). LRMS ($APCI^+$) calcd for $C_{14}H_{14}NO_4S$ 292 (MH^+), found 292.

4.1.31. 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiazole-5-carbaldehyde (50)

Compound **31** was deprotected according to general procedure E to give **50** as a cream solid (100%). ^1H NMR [$(CD_3)_2SO$] δ 10.13 (s, 1H), 8.86 (s, 1H), 8.40 (d, J = 0.7 Hz, 1H), 8.27 (dt, J = 8.0, 0.7 Hz, 1H), 8.01 (d, J = 8.2 Hz, 1H), 5.51 (s, 2H). HRMS (FAB^+) calcd for $C_{12}H_8NO_3S$ 246.0225 (MH^+), found 246.0224.

4.1.32. (*E,Z*)-5-((2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiazol-5-yl)methylene)-2-thioxoimidazolidin-4-one (103)

Reaction of **50** with 2-thiohydantoin according to general procedure D gave **103** as a yellow solid (89%); mp (AcOH) >300 °C. ^1H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.14–12.57 (m, 2H), 8.58 (d, J = 0.6 Hz, 0.7H), 8.48 (d, J = 0.5 Hz, 0.3H), 8.26–8.30 (m, 1H), 8.12–8.19 (m, 1H), 7.95–8.01 (m, 1H), 6.94 (s, 0.3H), 6.82 (s, 0.7H), 5.50 (s, 1.4H), 5.49 (s, 0.6H). LRMS ($APCI^-$) calcd for $C_{15}H_8N_3O_3S_2$ 342 (M−H), found 342. Anal. ($C_{15}H_9N_3O_3S_2{\cdot}0.25H_2O$) C, H, N.

4.1.33. 5-(2-(Dimethoxymethyl)thiazol-4-yl)isobenzofuran-1(3*H*)-one (32)

4-Bromo-2-formyl-1,3-thiazole was protected as the dimethyl acetal **12** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **32** as a off-white solid (16%). ^1H NMR [$(CD_3)_2SO$] δ 8.27 (d, J = 0.6 Hz, 1H), 8.14 (dt, J = 8.0, 0.8 Hz, 1H), 7.96 (dd, J = 8.0, 0.4 Hz, 1H), 7.84 (d, J = 0.7 Hz, 1H), 5.59 (d, J = 0.7 Hz, 1H), 5.48 (s, 2H), 3.34 (s, 6H). LRMS ($APCI^+$) calcd for $C_{14}H_{14}NO_4S$ 292 (MH^+), found 292.

4.1.34. 4-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiazole-2-carbaldehyde (51)

Compound **32** was deprotected according to general procedure E to give **51** as a white solid (100%). ^1H NMR [$(CD_3)_2SO$] δ 10.02 (s, 1H), 8.88 (s, 1H), 8.35 (s, 1H), 8.23 (d, J = 8.0 Hz, 1H), 8.00 (d, J = 8.0 Hz, 1H), 5.50 (s, 2H). HRMS (ESI-TOF) calcd for $C_{12}H_8NO_3SNa$ 268.0044 (M+Na), found 268.0039.

4.1.35. (*E,Z*)-5-((4-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiazol-2-yl)methylene)-2-thioxoimidazolidin-4-one (104)

Reaction of **51** with 2-thiohydantoin according to general procedure D gave **104** as a yellow solid (73%); mp (AcOH) >295 °C. ^1H NMR [$(CD_3)_2SO$] δ 12.45 (br s, 1H), 11.26 (br s, 1H), 8.43 (s, 1H), 8.38 (dd, J = 8.0, 1.4 Hz, 1H), 8.35 (s, 1H), 8.02 (d, J = 7.9 Hz, 1H), 6.66 (s, 1H), 5.53 (s, 2H). LRMS ($APCI^-$) calcd for $C_{15}H_8N_3O_3S_2$ 342 (M−H), found 342. Anal. ($C_{15}H_9N_3O_3S_2$) C, H, N.

4.1.36. 5-(4-(Dimethoxymethyl)thiazol-2-yl)isobenzofuran-1(3*H*)-one (33)

2-Bromo-1,3-thiazole-4-carboxaldehyde was protected as the dimethyl acetal **13** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **33** as a pale pink solid (51%). ^1H NMR [$(CD_3)_2SO$] δ 8.45 (s, 1H), 8.26 (s, 1H), 8.18 (dd, J = 8.1, 1.2 Hz, 1H), 7.92 (d, J = 8.0 Hz, 1H), 5.72 (s, 1H), 5.47 (s, 2H), 3.41 (s, 6H). LRMS ($APCI^+$) calcd for $C_{14}H_{14}NO_4S$ 292 (MH^+), found 292.

4.1.37. 2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiazole-4-carbaldehyde (52)

Compound **33** was deprotected according to general procedure E to give **52** as a white solid (100%). ^1H NMR [$(CD_3)_2SO$] δ 10.05 (d, J = 1.2 Hz, 1H), 8.92 (d, J = 1.2 Hz, 1H), 8.34–8.36 (m, 1H), 8.26–8.29 (m, 1H), 7.98 (d, J = 7.9 Hz, 1H), 5.50 (s, 2H). HRMS (ESI-TOF) calcd for $C_{12}H_7NO_3SNa$ 268.0044 (M+Na), found 268.0039.

4.1.38. (*E,Z*)-5-((2-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiazol-4-yl)methylene)-2-thioxoimidazolidin-4-one (105)

Reaction of **52** with 2-thiohydantoin according to general procedure D gave **105** as a yellow-brown solid (88%); mp (AcOH) >295 °C. ^1H NMR [$(CD_3)_2SO$] δ 12.62 (br s, 1H), 11.43 (br s, 1H), 8.83 (s, 1H), 8.54 (s, 1H), 8.37 (dd, J = 8.1, 1.2 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 6.84 (s, 1H), 5.52 (s, 2H). LRMS ($APCI^-$) calcd for $C_{15}H_8N_3O_3S_2$ 342 (M−H), found 342. Anal. ($C_{15}H_9N_3O_3S_2$) C, H, N.

4.1.39. General procedure F: 5-(hydroxymethyl)isobenzofuran-1(3*H*)-one (66)

Compound **65** (3.00 g, 16.8 mmol) was dissolved in THF (300 mL), to which was added carbonyldiimidazole (CDI; 5.46 g, 33.7 mmol). After stirring at rt for 2 h., this reaction mixture was slowly added to a solution of $NaBH_4$ (3.18 g, 84.0 mmol) in water (80 mL), stirred for a further 5 minutes, then quenched with concd HCl to pH 2. This aqueous mixture was extracted with EtOAc (2 × 50 mL), the fractions combined, dried (Na_2SO_4) and filtered, then the solvent removed under reduced pressure to afford a crude solid which was purified by flash column chromatography on silica gel (EtOAc as eluant). The title compound **66** was isolated as a white solid (2.59 g, 94%). ^1H NMR in agreement with literature values.[45]

4.1.40. General procedure G: 1-oxo-1,3-dihydroisobenzofuran-5-carbaldehyde (67)

Compound **66** (1.0 g, 6.10 mmol) was dissolved in CH_2Cl_2 (200 mL), pyridinium chlorochromate on silica gel[46] (6.71 g of 1 mmol/g silica) added, and the mixture stirred at rt for 3 h. All solvent was then removed under reduced pressure and the resulting powder loaded onto a column of flash silica gel which was eluted with CH_2Cl_2 to obtain **67** as a white solid (620 mg, 63%). ^{1}H NMR in agreement with literature values.[45,47]

4.1.41. (*E,Z*)-1-Oxo-1,3-dihydroisobenzofuran-5-carbaldehyde oxime (68)

Compound **67** (445 mg, 2.75 mmol) was dissolved in MeOH (40 mL), to which was added pyridine (1.09 g, 13.7 mmol) and $NH_2OH{\cdot}HCl$ (954 mg, 13.7 mmol). The reaction mixture was stirred at 60 °C for 1.5 h, then upon cooling was diluted with water (100 mL), with the resulting white precipitate collected by filtration and dried to give **68** (325 mg, 67%). ^{1}H NMR in agreement with literature values.[47]

4.1.42. 5-(5-(Hydroxymethyl)isoxazol-3-yl)isobenzofuran-1(3*H*)-one (69)

Compound **68** (320 mg, 1.81 mmol) was dissolved in THF (10 mL) then pyridine (1 drop) and *N*-chlorosuccinimide (266 mg, 1.99 mmol) added. This mixture was heated at 60 °C for 0.5 h. Upon cooling to rt, propargyl alcohol (132 mg, 2.35 mmol) and triethylamine (201 mg, 1.99 mmol) were added and a white precipitate formed. The reaction was then heated at 50 °C for 2 h. The solvent was removed under reduced pressure and the residue taken up into CH_2Cl_2 (100 mL), which was then washed with water (2 × 100 mL), brine (100 mL) and dried (Na_2SO_4). Removal of the solvent afforded a white solid which was purified by flash column chromatography on silica gel (10% acetone/CH_2Cl_2 as eluant) to give **69** as a fluffy white solid (56%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.19 (d, J = 0.4 Hz, 1H), 8.04 (dt, J = 8.0, 0.7 Hz, 1H), 7.97 (d, J = 8.1 Hz, 1H), 7.05 (s, 1H), 5.74 (t, J = 6.0 Hz, 1H), 5.49 (s, 2H), 4.65 (d, J = 5.7 Hz, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_{10}NO_4$ 232 (MH^+), found 232.

4.1.43. 3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)isoxazole-5-carbaldehyde (70)

Compound **69** was oxidized to the corresponding aldehyde according to general procedure G. Purification by flash column chromatography on silica gel (CH_2Cl_2 as eluant) gave **70** as a white solid (65%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.00 (s, 1H), 8.30 (s, 1H), 8.18 (dt, J = 8.0, 0.7 Hz, 1H), 8.08 (s, 1H), 8.03 (d, J = 8.0 Hz, 1H), 5.52 (s, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_8NO_4$ 230 (MH^+), found 230.

4.1.44. (*E,Z*)-5-((3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)isoxazol-5-yl)methylene)-2-thioxoimidazolidin-4-one (106)

Reaction of **70** with 2-thiohydantoin according to general procedure D gave **106** as a yellow solid (86%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.65 (br s, 1H), 12.29 (br s, 1H), 8.19 (br s, 0.1H), 8.17 (br s, 0.9H), 8.03–8.09 (m, 2H), 7.95 (s, 0.1H), 7.88 (s, 0.9H), 6.50 (s, 0.1H), 6.44 (s, 0.9H), 5.52 (s, 1.8H), 5.50 (s, 0.2H). LRMS (APCI$^-$) calcd for $C_{15}H_8N_3O_4S$ 326 (M−H), found 326. Anal. ($C_{15}H_9N_3O_4S{\cdot}0.5H_2O$) C, H, N.

4.1.45. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1,3,4-oxathiazol-2-one (76)

Compound **75** (230 mg, 1.30 mmol) was suspended in dioxane (20 mL), chlorocarbonylsulfenyl chloride (340 mg, 2.60 mmol) added, and the mixture heated at reflux for 15 h. Upon cooling, all solvent was removed under reduced pressure and the resulting solid purified by flash column chromatography on silica gel (CH_2Cl_2 as eluant) to give **76** as a cream solid (244 mg, 80%); mp (CH_2Cl_2/hexane) 195–198 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.24 (dd, J = 1.1, 0.7 Hz, 1H), 8.11 (dt, J = 8.0, 0.7 Hz, 1H), 8.02 (dd, J = 8.1, 0.4 Hz, 1H), 5.50 (s, 2H). LRMS (APCI$^+$) calcd for $C_{10}H_6NO_4S$ 236 (MH^+), found 236. Anal. ($C_{10}H_5NO_4S$) C, H, N.

4.1.46. Ethyl 3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)isothiazole-4-carboxylate (77)

A solution of **76** (240 mg, 1.02 mmol) and ethyl propiolate (400 mg, 4.08 mmol) in 1,2-dichlorobenzene (2 mL) was heated at 150 °C for 15 h. All solvent was removed under reduced pressure and the resulting mixture of two isomers (273 mg, 93% total yield) partially separated by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant). The title compound **77** was isolated as a white solid (53 mg, 18%). ^{1}H NMR [$(CD_3)_2SO$] δ 9.78 (s, 1H), 7.91 (d, J = 8.0 Hz, 1H), 7.88 (br s, 1H), 7.77 (dt, J = 7.9, 0.6 Hz, 1H), 5.48 (s, 2H), 4.21 (q, J = 7.1 Hz, 2H), 1.18 (t, J = 7.1 Hz, 3H). LRMS (APCI$^+$) calcd for $C_{14}H_{12}NO_4S$ 290 (MH^+), found 290.

4.1.47. General procedure H: 3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)isothiazole-4-carboxylic acid (78)

Compound **77** (53 mg, 0.18 mmol) was suspended in EtOH (10 mL), to which was added 2 M NaOH (10 mL). This mixture was stirred at 50 °C for 4 h., allowed to cool, and partitioned between 1 M HCl (20 mL) and EtOAc (20 mL). The aqueous layer was further extracted with EtOAc (2 × 20 mL), then the combined organic fractions dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure. The resulting crude solid was dissolved in 1:1 TFA/CH_2Cl_2 (4 mL) and stirred at rt for 4 h. Removal of the reaction solvent under reduced pressure gave **78** as a white solid (47 mg, 98%) which was isolated without further purification. ^{1}H NMR [$(CD_3)_2SO$] δ 13.15 (br s, 1H), 9.72 (s, 1H), 7.90 (d, J = 8.0 Hz, 1H), 7.87 (s, 1H), 7.77 (dt, J = 7.9, 0.7 Hz, 1H), 5.47 (s, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_8NO_4S$ 262 (MH^+), found 262.

4.1.48. 5-(4-(Hydroxymethyl)isothiazol-3-yl)isobenzofuran-1(3*H*)-one (79)

Compound **78** was reduced according to general procedure F to give **79** as a pale yellow waxy solid (37 mg, 84%). ^{1}H NMR [$(CD_3)_2SO$] δ 9.05 (s, 1H), 8.06 (s, 1H), 8.00 (dt, J = 8.0, 0.7 Hz, 1H), 7.95 (d, J = 7.9 Hz, 1H), 5.49 (br s, 3H), 4.64 (d, J = 0.6 Hz, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_{10}NO_3S$ 248 (MH^+), found 248.

4.1.49. 3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)isothiazole-4-carbaldehyde (80)

Compound **79** was oxidized to the corresponding aldehyde according to general procedure G to give **80** as a white solid (84%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.01 (s, 1H), 9.98 (s, 1H), 8.00–8.02 (m, 1H), 7.97 (d, J = 8.0 Hz, 1H), 7.92 (dt, J = 7.9, 0.7 Hz, 1H), 5.50 (s, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_8NO_3S$ 246 (MH^+), found 246.

4.1.50. (*E,Z*)-5-((3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)isothiazol-4-yl)methylene)-2-thioxoimidazolidin-4-one (107)

Reaction of **80** with 2-thiohydantoin according to general procedure D gave **107** as a yellow solid (86%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.32 (br s, 2H), 9.70 (d, J = 0.5 Hz, 1H), 8.00 (d, J = 7.9 Hz, 1H), 7.93 (s, 1H), 7.81 (dt, J = 8.0, 0.7 Hz, 1H), 6.25 (s, 1H), 5.49 (s, 2H). LRMS (APCI$^-$) calcd for $C_{15}H_8N_3O_3S_2$ 342 (M−H), found 342. Anal. ($C_{15}H_9N_3O_3S_2$) C, H, N.

4.1.51. Ethyl 3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)isothiazole-5-carboxylate (81)

The mixture of two isomers (273 mg, 93% total yield) isolated above was partially separated by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant). The title compound **81** was isolated as a white solid (119 mg, 40%). ^{1}H NMR [$(CD_3)_2SO$]

δ 8.65 (s, 1H), 8.42 (s, 1H), 8.33 (dt, J = 8.1, 0.7 Hz, 1H), 7.96 (d, J = 8.1 Hz, 1H), 5.49 (s, 2H), 4.40 (q, J = 7.1 Hz, 2H), 1.36 (t, J = 7.1 Hz, 3H). LRMS (APCI$^+$) calcd for $C_{14}H_{12}NO_4S$ 290 (MH^+), found 290.

4.1.52. 5-(5-(Hydroxymethyl)isothiazol-3-yl)isobenzofuran-1(3*H*)-one (83)

Compound **81** was deprotected to the corresponding carboxylic acid, 3-(1-oxo-1,3-dihydroisobenzofuran-5-yl)isothiazole-5-carboxylic acid **82**, according to general procedure H. Without further purification, the crude acid was then reduced according to general procedure F to give **83** as a yellow-white solid (49%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.28 (s, 1H), 8.20 (dt, J = 8.0, 0.7 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.87 (t, J = 1.0 Hz, 1H), 5.97 (t, J = 5.6 Hz, 1H), 5.48 (s, 2H), 4.90 (dd, J = 5.6, 1.0 Hz, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_{10}NO_3S$ 248 (MH^+), found 248.

4.1.53. 3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)isothiazole-5-carbaldehyde (84)

Compound **83** was oxidized according to general procedure G to give **84** as a white solid (48%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.21 (s, 1H), 8.78 (s, 1H), 8.38 (t, J = 0.7 Hz, 1H), 8.29 (dt, J = 8.0, 0.7 Hz, 1H), 8.00 (d, J = 8.0 Hz, 1H), 5.51 (s, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_8NO_3S$ 246 (MH^+), found 246.

4.1.54. (*E,Z*)-5-((3-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)isothiazol-5-yl)methylene)-2-thioxoimidazolidin-4-one (108)

Reaction of **84** with 2-thiohydantoin according to general procedure D gave **108** as a mustard-yellow solid (79%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.59 (br s, 1H), 12.42 (br s, 0.3H), 12.33 (br s, 0.7H), 8.60 (s, 0.7H), 8.42 (s, 0.3H), 8.34 (s, 0.7H), 8.28–8.33 (m, 1H), 8.21 (dd, J = 8.1, 1.2 Hz, 0.3H), 8.02 (d, J = 8.0 Hz, 0.7H), 7.96 (d, J = 8.0 Hz, 0.3H), 6.97 (s, 0.3H), 6.83 (s, 0.7H), 5.52 (s, 1.4H), 5.49 (s, 0.6H). LRMS (APCI$^-$) calcd for $C_{15}H_8N_3O_3S_2$ 342 (M−H), found 342. Anal. ($C_{15}H_9N_3O_3S_2$) C, H, N.

4.1.55. 5-(3-(Dimethoxymethyl)benzo[*b*]thiophen-5-yl)isobenzofuran-1(3*H*)-one (34)

5-Bromobenzothiophene-3-carboxaldehyde was protected as the dimethyl acetal **14** according to a literature procedure,[44] then reacted with **22** according to general procedure A. Purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant) gave **34** as a white solid (56%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.25 (d, J = 1.5 Hz, 1H), 8.16 (d, J = 8.5 Hz, 1H), 8.01 (d, J = 0.4 Hz, 1H), 7.96 (d, J = 8.1 Hz, 1H), 7.92 (dd, J = 8.1, 1.4 Hz, 1H), 7.84 (s, 1H), 7.77 (dd, J = 8.4, 1.8 Hz, 1H), 5.84 (s, 1H), 5.50 (s, 2H), 3.30 (s, 6H). LRMS (APCI$^+$) calcd for $C_{19}H_{17}O_4S$ 341 (MH^+), found 341.

4.1.56. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzo[*b*]thiophene-3-carbaldehyde (53)

Compound **34** was deprotected according to general procedure E to give **53** as a white solid (100%). ^{1}H NMR [$(CD_3)_2SO$] δ 10.19 (s, 1H), 9.07 (s, 1H), 8.88 (d, J = 1.5 Hz, 1H), 8.28 (d, J = 8.0 Hz, 1H), 8.04 (br s, 1H), 7.92–8.00 (m, 2H), 7.89 (dd, J = 8.5, 1.9 Hz, 1H), 5.51 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{11}O_3S$ 295 (MH^+), found 295.

4.1.57. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzo[*b*]thiophen-3-yl)methylene)-2-thioxoimidazolidin-4-one (109)

Reaction of **53** with 2-thiohydantoin according to general procedure D gave **109** as a yellow solid (97%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.28 (br s, 2H), 8.56 (s, 1H), 8.41 (d, J = 1.5 Hz, 1H), 8.22 (d, J = 8.5 Hz, 1H), 8.16 (d, J = 0.7 Hz, 1H), 8.07 (dd, J = 8.0, 1.4 Hz, 1H), 7.94 (d, J = 8.0 Hz, 1H), 7.86 (dd, J = 8.5, 1.7 Hz, 1H), 6.95 (s, 1H), 5.50 (s, 2H). Anal. ($C_{20}H_{12}N_2O_3S_2 \cdot 0.5H_2O$) C, H, N.

4.1.58. 5-Bromo-2-(dimethoxymethyl)benzo[*b*]thiophene (15)

5-Bromo-2-methylbenzo[*b*]thiophene[35] was oxidized to 5-bromobenzo[*b*]thiophene-2-carbaldehyde using a previously reported method.[36] This aldehyde was then protected as the dimethyl acetal according a literature procedure[44] to give **15** as a light orange solid (44%). ^{1}H NMR ($CDCl_3$) δ 7.89 (d, J = 1.8 Hz, 1H), 7.67 (d, J = 8.6 Hz, 1H), 7.41 (dd, J = 8.6, 1.8 Hz, 1H), 7.25 (s, 1H), 5.68 (d, J = 1.0 Hz, 1H), 3.41 (s, 6H). LRMS (APCI$^+$) calcd for $C_{10}H_8BrOS$ 255 (M^+−MeOH), found 255.

4.1.59. 5-(2-(Dimethoxymethyl)benzo[*b*]thiophen-5-yl)isobenzofuran-1(3*H*)-one (35)

Compound **15** was reacted with **22** according to general procedure A. The title compound **35** was obtained as a white solid (56%). ^{1}H NMR ($CDCl_3$) δ 8.00 (d, J = 8.6 Hz, 1H), 7.98 (s, 1H), 7.93 (d, J = 8.4 Hz, 1H), 7.80 (dt, J = 8.0, 0.7 Hz, 1H), 7.72 (dd, J = 1.2, 0.7 Hz, 1H), 7.57 (dd, J = 8.4, 1.8 Hz, 1H), 7.40 (s, 1H), 5.73 (d, J = 1.0 Hz, 1H), 5.39 (s, 2H), 3.44 (s, 6H). LRMS (APCI$^+$) calcd for $C_{19}H_{17}O_4S$ 341 (MH^+), found 341.

4.1.60. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzo[*b*]thiophene-2-carbaldehyde (54)

Compound **35** was deprotected according to general procedure E to give **54** as a white solid (0.19 g, 99%). ^{1}H NMR ($CDCl_3$) δ 10.16 (s, 1H), 8.17 (d, J = 1.5 Hz, 1H), 8.11 (s, 1H), 8.03 (d, J = 7.9 Hz, 1H), 8.02 (d, J = 8.5 Hz, 1H), 7.81 (dt, J = 8.0, 0.7 Hz, 1H), 7.76 (dd, J = 8.5, 1.8 Hz, 1H), 7.74 (dd, J = 1.4, 0.7 Hz, 1H), 5.41 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{11}O_3S$ 295 (MH^+), found 295.

4.1.61. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzo[*b*]thiophen-2-yl)methylene)-2-thioxoimidazolidin-4-one (110)

Reaction of **54** with 2-thiohydantoin was carried out according to general procedure D to give **110** as a bright yellow solid (90%); mp (AcOH) >305 °C. ^{1}H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.03–12.60 (br m, 2H), 8.20 (d, J = 1.6 Hz, 1H), 8.19 (s, 1H), 8.14 (d, J = 8.5 Hz, 1H), 8.11 (s, 0.1H), 8.04 (s, 0.9H), 7.95–7.99 (m, 2H), 7.79 (dd, J = 8.5, 1.8 Hz, 1H), 6.92 (s, 0.1H), 6.66 (s, 0.9H), 5.50 (s, 2H). HRMS (ESI$^-$) calcd for $C_{20}H_{11}N_2O_3S_2$ 391.0217 (M−H), found 391.0218. Anal. ($C_{20}H_{12}N_2O_3S_2$) C, H, N.

4.1.62. 6-Bromo-2-(dimethoxymethyl)benzo[*b*]thiophene (16)

6-Bromo-2-methylbenzo[*b*]thiophene[35] was oxidized to 6-bromobenzo[*b*]thiophene-2-carbaldehyde using a previously reported method.[36] This aldehyde was then protected as the dimethyl acetal according a literature procedure[44] to give **16** as a yellow solid (61%). ^{1}H NMR ($CDCl_3$) δ 7.96 (d, J = 1.8 Hz, 1H), 7.59 (d, J = 8.5 Hz, 1H), 7.44 (dd, J = 8.5, 1.8 Hz, 1H), 7.27 (s, 1H), 5.67 (d, J = 1.0 Hz, 1H), 3.41 (s, 6H).

4.1.63. 5-(2-(Dimethoxymethyl)benzo[*b*]thiophen-6-yl)isobenzofuran-1(3*H*)-one (36)

Compound **16** was reacted with **22** according to general procedure A to give **36** as a white solid (72%). ^{1}H NMR ($CDCl_3$) δ 8.08 (d, J = 0.8 Hz, 1H), 8.00 (d, J = 8.0 Hz, 1H), 7.85 (d, J = 8.3 Hz, 1H), 7.81 (dd, J = 8.0, 0.7 Hz, 1H), 7.73 (s, 1H), 7.60 (dd, J = 8.3, 1.6 Hz, 1H), 7.37 (s, 1H), 5.73 (s, 1H), 5.39 (s, 2H), 3.44 (s, 6H). LRMS (APCI$^+$) calcd for $C_{19}H_{17}O_4S$ 341 (MH^+), found 341.

4.1.64. 6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzo[*b*]thiophene-2-carbaldehyde (55)

Compound **36** was deprotected according to general procedure E to give **55** as a white solid (76%). ^{1}H NMR ($CDCl_3$) δ 10.15 (s, 1H), 8.15 (d, J = 1.5 Hz, 1H), 8.08 (s, 1H), 8.06 (d, J = 8.4 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.83 (dd, J = 8.0, 1.2 Hz, 1H), 7.76 (s, 1H), 7.70 (dd, J = 8.4, 1.6 Hz, 1H), 5.40 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{11}O_3S$ 295 (MH^+), found 295.

4.1.65. (*E,Z*)-5-((6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzo[*b*]thiophen-2-yl)methylene)-2-thioxoimidazolidin-4-one (111)

Reaction of **55**with 2-thiohydantoin was carried out according to general procedure D to give **111** as a brown solid (40%); mp (AcOH) >310 °C. ^{1}H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.05–12.58 (br m, 2H), 8.44 (d, J = 1.5 Hz, 1H), 8.17 (s, 1H), 8.07 (s, 1H), 7.94–8.01 (m, 3H), 7.82 (dd, J = 8.4, 1.7 Hz, 1H), 6.93 (s, 0.05H), 6.64 (s, 0.95H), 5.49 (s, 2H). HRMS (ESI$^-$) calcd for $C_{20}H_{11}N_2O_3S_2$ 391.0217 (M−H), found 391.0213.Anal. ($C_{20}H_{12}N_2O_3S_2$·0.2AcOH) C, H, N.

4.1.66. 5-Bromo-2-(dimethoxymethyl)benzofuran (17)

5-Bromobenzofuran-2-carbaldehydewas protected as the dimethyl acetal according to a literature procedure[44] to give **17** as a yellow solid (98%). ^{1}H NMR ($CDCl_3$) δ 7.70 (d, J = 1.5 Hz, 1H), 7.35–7.40 (m, 2H), 6.76 (s, 1H), 5.55 (s, 1H), 3.41 (s, 6H). LRMS (APCI$^+$) calcd for $C_{10}H_8BrO_2$ 239 (M^+−MeOH), found 239.

4.1.67. 5-(2-(Dimethoxymethyl)benzofuran-5-yl)isobenzofuran-1(3*H*)-one (37)

Compound **17** was reacted with **22** according to general procedure A to give **37** as a white solid (53%). ^{1}H NMR ($CDCl_3$) δ 7.99 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 1.8 Hz, 1H), 7.77 (dd, J = 8.0, 1.4 Hz, 1H), 7.68 (d, J = 0.6 Hz, 1H), 7.60 (d, J = 8.6 Hz, 1H), 7.54 (dd, J = 8.6, 1.9 Hz, 1H), 6.76 (t, J = 0.8 Hz, 1H), 5.60 (d, J = 0.8 Hz, 1H), 5.38 (s, 2H), 3.45 (s, 6H). LRMS (APCI$^+$) calcd for $C_{19}H_{17}O_5$ 325 (MH^+), found 325.

4.1.68. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzofuran-2-carbaldehyde (56)

Compound **37** was deprotected according to general procedure E to give **56** as a white solid (80%). ^{1}H NMR ($CDCl_3$) δ 9.93 (s, 1H), 7.98–8.03 (m, 2H), 7.69–7.79 (m, 4H), 7.63 (d, J = 0.8 Hz, 1H), 5.40 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{11}O_4$ 279 (MH^+), found 279.

4.1.69. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)benzofuran-2-yl)methylene)-2-thioxoimidazolidin-4-one (112)

Reaction of **56** with 2-thiohydantoin was carried out according to general procedure D to give **112** as a bright yellow solid (87%); mp (AcOH) >310 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.23 (br s, 2H), 8.06 (d, J = 0.9 Hz, 1H), 8.00 (s, 1H), 7.93 (d, J = 0.8 Hz, 2H), 7.77–7.82 (m, 2H), 7.50 (s, 1H), 6.56 (s, 1H), 5.48 (s, 2H). HRMS (ESI$^-$) calcd for $C_{20}H_{11}N_2O_4S$ 375.0445 (M−H), found 375.0435. Anal. ($C_{20}H_{12}N_2O_4S$·0.10AcOH) C, H, N.

4.1.70. 6-Bromo-2-(dimethoxymethyl)-1*H*-indole (18)

6-Bromo-1*H*-indole-2-carbaldehydewas protected as the dimethyl acetal according to a literature procedure[44] to give **18** as a yellow solid (81%). ^{1}H NMR ($CDCl_3$) δ 8.36 (br s, 1H), 7.51 (s, 1H), 7.45 (d, J = 8.4 Hz, 1H), 7.20 (dd, J = 8.4, 1.7 Hz, 1H), 6.48–6.51 (m, 1H), 5.61 (d, J = 0.6 Hz, 1H), 3.38 (s, 6H). LRMS (APCI$^+$) calcd for $C_{10}H_9BrNO$ 238 (M^+−MeOH), found 238.

4.1.71. 5-(2-(Dimethoxymethyl)-1*H*-indol-6-yl)isobenzofuran-1(3*H*)-one (38)

Compound **18** was reacted with **22** according to general procedure A to give **38** as a pale yellow solid (29%). ^{1}H NMR ($CDCl_3$) δ 8.53 (br s, 1H), 7.97 (d, J = 8.1 Hz, 1H), 7.79 (d, J = 7.9 Hz, 1H), 7.68–7.73 (m, 2H), 7.61 (t, J = 0.8 Hz, 1H), 7.39 (dd, J = 8.2, 1.6 Hz, 1H), 6.58 (t, J = 1.0 Hz, 1H), 5.67 (d, J = 0.5 Hz, 1H), 5.37 (s, 2H), 3.41 (s, 6H). LRMS (APCI$^+$) calcd for $C_{19}H_{18}NO_4$ 324 (MH^+), found 324.

4.1.72. 6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-indole-2-carbaldehyde (57)

Compound **38** was deprotected according to general procedure E to give **57** as a pale yellow solid (69%). ^{1}H NMR ($CDCl_3$) δ 9.89 (s, 1H), 9.12 (br s, 1H), 8.01 (d, J = 8.0 Hz, 1H), 7.87 (d, J = 8.4 Hz, 1H), 7.81 (d, J = 7.5 Hz, 1H), 7.73 (s, 1H), 7.67 (s, 1H), 7.46 (dd, J = 8.4, 1.5 Hz, 1H), 7.32 (q, J = 0.9 Hz, 1H), 5.39 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{12}NO_3$ 278 (MH^+), found 278.

4.1.73. (*E,Z*)-5-((6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-indol-2-yl)methylene)-2-thioxoimidazolidin-4-one (113)

Reaction of **57** with 2-thiohydantoin was carried out according to general procedure D to give **113** as a bright red solid (74%); mp (AcOH) >310 °C. ^{1}H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.10–12.79 (br m, 2H), 12.03 (s, 0.8H), 11.591 (s, 0.2H), 7.90–8.02 (m, 4H), 7.74 (s, 0.2H), 7.73 (d, J = 8.4 Hz, 0.8H), 7.48 (dd, J = 8.4, 1.6 Hz, 0.8H), 7.47 (dd, J = 8.3, 1.7 Hz, 0.2H), 7.38 (s, 0.2H), 7.16 (s, 0.8H), 6.72 (s, 0.8H), 6.59 (s, 0.2H), 5.47 (s, 2H). HRMS (ESI$^-$) calcd for $C_{20}H_{12}N_3O_3S$ 374.0605 (M−H), found 374.0608. Anal. ($C_{20}H_{13}N_3O_3S$·0.25AcOH) C, H, N.

4.1.74. 5-Bromo-2-(dimethoxymethyl)-1*H*-indole (19)

5-Bromo-1*H*-indole-2-carbaldehyde was protected as the dimethyl acetal according to a literature procedure[44] to give **19** as an orange solid (95%). ^{1}H NMR ($CDCl_3$) δ 8.42 (br s, 1H), 7.73 (t, J = 0.9 Hz, 1H), 7.20–7.28 (m, 2H), 6.45–6.48 (m, 1H), 5.62 (d, J = 0.6 Hz, 1H), 3.40 (s, 6H). LRMS (APCI$^+$) calcd for $C_{10}H_8BrO_2$ 239 (M^+−MeOH), found 239.

4.1.75. 5-(2-(Dimethoxymethyl)-1*H*-indol-5-yl)isobenzofuran-1(3*H*)-one (39)

Compound **19** was reacted with **22** according to general procedure A to give **39** as a pale red solid (67%). ^{1}H NMR ($CDCl_3$) δ 8.50 (br s, 1H), 7.96 (d, J = 8.0 Hz, 1H), 7.86 (s, 1H), 7.80 (d, J = 8.0 Hz, 1H), 7.71 (s, 1H), 7.47 (s, 2H), 6.61 (d, J = 1.8 Hz, 1H), 5.67 (s, 1H), 5.37 (s, 2H), 3.41 (s, 6H). LRMS (APCI$^+$) calcd for $C_{19}H_{18}NO_4$ 324 (MH^+), found 324.

4.1.76. 5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-indole-2-carbaldehyde (58)

Compound **39** was deprotected according to general procedure E to give **58** as a pale yellow solid (94%). ^{1}H NMR ($CDCl_3$) δ 12.12 (br s, 1H), 9.90 (s, 1H), 8.17 (d, J = 1.2 Hz, 1H), 7.98 (s, 1H), 7.92 (s, 2H), 7.76 (dd, J = 8.7, 1.8 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 7.49 (d, J = 0.8 Hz, 1H), 5.47 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{12}NO_3$ 278 (MH^+), found 278.

4.1.77. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)-1*H*-indol-2-yl)methylene)-2-thioxoimidazolidin-4-one (114)

Reaction of **58** with 2-thiohydantoin was carried out according to general procedure D to give **114** as a orange-brown solid (78%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] Observe *E*- and *Z*-isomers separately. δ 12.09–12.74 (br m, 2H), 12.03 (s, 0.8H), 11.591 (s, 0.2H), 7.90–8.03 (m, 4H), 7.74 (s, 0.2H), 7.72 (d, J = 8.4 Hz, 0.8H), 7.48 (dd, J = 8.4, 1.6 Hz, 0.8H), 7.46 (dd, J = 8.3, 1.6 Hz, 0.2H), 7.37 (s, 0.2H), 7.16 (s, 0.8H), 6.72 (s, 0.8H), 6.59 (s, 0.2H), 5.47 (s, 2H). HRMS (ESI$^-$) calcd for $C_{20}H_{12}N_3O_3S$ 374.0605 (M−H), found 374.0597. Anal. ($C_{20}H_{13}N_3O_3S$·0.20AcOH) C, H, N.

4.1.78. 7-Bromo-2-(dimethoxymethyl)quinoline (20)

7-Bromoquinoline-2-carbaldehyde was protected as the dimethyl acetal according to a literature procedure[44] to give **20** as a brown solid (90%). ^{1}H NMR ($CDCl_3$) δ 8.35 (d, J = 1.8 Hz, 1H), 8.17 (d, J = 8.5 Hz, 1H), 7.70 (d, J = 8.7 Hz, 1H), 7.69 (d, J = 8.5 Hz, 1H), 7.64 (dd, J = 8.7, 1.9 Hz, 1H), 5.46 (s, 1H), 3.47 (s, 6H). LRMS (APCI$^+$) calcd for $C_{12}H_{13}BrNO_2$ 283 (MH^+), found 283.

4.1.79. 5-(2-(Dimethoxymethyl)quinolin-7-yl)isobenzofuran-1(3*H*)-one (40)

Compound **20** was reacted with **22** according to general procedure A to give **40** as a yellow solid (62%). ^{1}H NMR ($CDCl_3$) δ 8.44 (t,

J = 0.9 Hz, 1H), 8.26 (d, *J* = 8.3 Hz, 1H), 8.05 (d, *J* = 7.9 Hz, 1H), 7.96 (d, *J* = 8.5 Hz, 1H), 7.91 (dt, *J* = 8.0, 0.8 Hz, 1H), 7.82–7.85 (m, 2H), 7.73 (d, *J* = 8.5 Hz, 1H), 5.52 (s, 1H), 5.41 (s, 2H), 3.50 (s, 6H). LRMS (APCI$^+$) calcd for $C_{20}H_{18}NO_4$ 336 (MH^+), found 336.

4.1.80. 7-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)quinoline-2-carbaldehyde (59)

Compound **40** was deprotected according to general procedure E to give **59** as a pale yellow solid (61%). ^{1}H NMR ($CDCl_3$) δ 10.25 (s, 1H), 8.52 (t, *J* = 0.9 Hz, 1H), 8.38 (d, *J* = 8.4 Hz, 1H), 8.09 (d, *J* = 8.4 Hz, 1H), 8.08 (d, *J* = 8.0 Hz, 1H), 8.05 (d, *J* = 8.5 Hz, 1H), 7.97 (dd, *J* = 8.5, 1.8 Hz, 1H), 7.94 (d, *J* = 8.6 Hz, 1H), 7.87 (s, 1H), 5.43 (s, 2H). LRMS (APCI$^+$) calcd for $C_{18}H_{12}NO_3$ 290 (MH^+), found 290.

4.1.81. (*E,Z*)-5-((7-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)quinoline-2-yl)methylene (115)

Reaction of **59** with 2-thiohydantoin was carried out according to general procedure D to give **115** as a yellow-green solid (85%); mp (AcOH) >310 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.81 (br s, 2H), 8.77 (s, 1H), 8.47 (d, *J* = 8.4 Hz, 1H), 8.20 (s, 1H), 8.12 (dd, *J* = 8.6, 2.7 Hz, 2H), 8.03 (dd, *J* = 8.5, 2.9 Hz, 2H), 7.87 (d, *J* = 8.4 Hz, 1H), 6.74 (s, 1H), 5.54 (s, 2H). HRMS (ESI$^-$) calcd for $C_{21}H_{12}N_3O_3S$ 386.0605 (M−H), found 386.0600. Anal. ($C_{21}H_{13}N_3O_3S$·0.30AcOH) C, H, N.

4.1.82. 6-Bromo-2-(dimethoxymethyl)quinoline (21)

6-Bromoquinoline-2-carbaldehyde was protected as the dimethyl acetal according to a literature procedure[44] [except using HCl (g) as catalyst instead of *p*-TsOH] to give **21** as a dark orange solid (70%). ^{1}H NMR ($CDCl_3$) δ 8.11 (d, *J* = 8.6 Hz, 1H), 8.02 (d, *J* = 9.0 Hz, 1H), 7.99 (d, *J* = 2.2 Hz, 1H), 7.79 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.69 (d, *J* = 8.6 Hz, 1H), 5.46 (s, 1H), 3.47 (s, 6H). LRMS (APCI$^+$) calcd for $C_{12}H_{13}BrNO_2$ 283 (MH^+), found 283.

4.1.83. 5-(2-(Dimethoxymethyl)quinolin-6-yl)isobenzofuran-1(3*H*)-one (41)

Compound **21** was reacted with **22** according to general procedure A to give **41** as a pale yellow solid (84%). ^{1}H NMR ($CDCl_3$) δ 8.28 (dd, *J* = 8.6, 1.6 Hz, 2H), 8.07 (d, *J* = 2.0 Hz, 1H), 8.05 (d, *J* = 8.0 Hz, 1H), 7.99 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.88 (d, *J* = 8.0 Hz, 1H), 7.80 (s, 1H), 7.74 (d, *J* = 8.5 Hz, 1H), 5.52 (s, 1H), 5.41 (s, 2H), 3.50 (s, 6H). LRMS (APCI$^+$) calcd for $C_{20}H_{18}NO_4$ 336 (MH^+), found 336.

4.1.84. 6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)quinoline-2-carbaldehyde (60)

Compound **41** was deprotected according to general procedure E to give **60** as a pale yellow solid (78%). ^{1}H NMR ($CDCl_3$) δ 10.26 (s, 1H), 8.40 (d, *J* = 8.4 Hz, 1H), 8.39 (d, *J* = 8.7 Hz, 1H), 8.04–8.14 (m, 4H), 7.91 (dt, *J* = 8.0, 0.6 Hz, 1H), 7.83 (s, 1H), 5.43 (s, 2H). LRMS (APCI$^+$) calcd for $C_{18}H_{12}NO_3$ 290 (MH^+), found 290.

4.1.85. (*E,Z*)-5-((6-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)quinolin-2-yl)methylene)-2-thioxoimidazolidin-4-one (116)

Reaction of **60** with 2-thiohydantoin was carried out according to general procedure D to give **116** as a yellow-green solid (82%); mp (AcOH) >310 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.50 (br s, 1H), 11.86 (br s, 1H), 8.48 (t, *J* = 8.2 Hz, 2H), 8.41 (d, *J* = 2.1 Hz, 1H), 8.23 (dd, *J* = 8.9, 2.1 Hz, 1H), 8.16 (s, 1H), 8.09 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.99 (d, *J* = 8.0 Hz, 1H), 7.88 (d, *J* = 8.5 Hz, 1H), 6.74 (s, 1H), 5.51 (s, 2H). HRMS (ESI$^-$) calcd for $C_{21}H_{12}N_3O_3S$ 386.0605 (M−H), found 386.0609. Anal. ($C_{21}H_{13}N_3O_3S$) C, H, N.

4.1.86. *N*-(3-Hydroxypropyl)-1-oxo-1,3-dihydroisobenzofuran-5-carboxamide (73)

To a stirred solution of **65** (500 mg, 2.81 mmol) in dry DMF (30 mL) was added triethylamine (398 mg, 3.93 mmol), EDCI·HCl (700 mg, 3.65 mmol) and HOBt (493 mg, 3.65 mmol). 3-Amino-1-propanol (338 mg, 4.49 mmol) was then added in one portion and the whole mixture stirred at rt for 16 h. The reaction mixture was concentrated, diluted with $NaHCO_3$ (100 mL), and extracted with EtOAc (3 × 100 mL). The combined organic fractions were dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure to give a crude oil, which was dry-loaded onto silica gel. Flash column chromatography (EtOAc followed by 0.5–1% MeOH/EtOAc as eluant) yielded **73** as a white crystalline solid (343 mg, 52%); mp 130–132 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.68 (br t, *J* = 5.1 Hz, 1H), 8.07 (s, 1H), 8.00 (d, *J* = 8.0 Hz, 1H), 7.92 (d, *J* = 8.0 Hz, 1H), 5.46 (s, 2H), 4.45 (t, *J* = 5.2 Hz, 1H), 3.44–3.52 (m, 2H), 3.29–3.40 (m, 2H), 1.65–1.74 (m, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_{13}NO_4$ 236 (MH^+), found 236.

4.1.87. 1-Oxo-*N*-(3-oxopropyl)-1,3-dihydroisobenzofuran-5-carboxamide (74)

To a solution of **73** (118 mg, 0.50 mmol) in THF/DMF 1:1 (5 mL), Dess–Martin periodinane (319 mg, 0.75 mmol) was added. After stirring for 3 h. at rt, the solution was diluted with CH_2Cl_2 (15 mL), and washed with satd aq NaS_2O_3 (10 mL), $NaHCO_3$ (10 mL), and brine (10 mL). The organic extract was evaporated and further dried under high vacuum. Flash column chromatography (50% EtOAc/hexanes as eluant) yielded **74** as a off-white solid (101 mg, 86%) which was used directly in the next step. ^{1}H NMR [$(CD_3)_2SO$] δ 9.70 (t, *J* = 1.6 Hz, 1H), 8.81 (br t, *J* = 5.1 Hz, 1H), 8.06 (s, 1H),7.97 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 5.46 (s, 2H), 3.53–3.61 (m, 2H), 2.69–2.75 (m, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_{11}NO_4$ 234 (MH^+), found 234.

4.1.88. (*E,Z*)-1-Oxo-*N*-(3-(5-oxo-2-thioxoimidazolidin-4-ylidene)propyl)-1,3-dihydroisobenzofuran-5-carboxamide (117)

Reaction of **74** with 2-thiohydantoin was carried out according to general procedure D to give a crude product which was purified by preparative HPLC [Synergi-Max RP C12 column, eluting with a gradient composed of mobile phases A (1% water/TFA) and B (90% MeCN/water)]. The title compound **117** was isolated as a brown solid (10%); mp (AcOH) 239–243 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.10 (br s, 1H), 11.97 (br s, 1H), 8.86 (s, 1H), 8.06 (s, 1H), 7.98 (d, *J* = 8.1 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 1H), 5.74 (t, *J* = 7.8 Hz, 1H), 5.47 (s, 2H), 3.38–3.48 (m, 2H), 2.52–2.60 (m, 2H).HRMS (ESI$^-$) calcd for $C_{20}H_{11}N_2O_4S$ 330.0554 (M−H), found 330.0546.

4.1.89. 5-(Thiophen-2-yl)isobenzofuran-1(3*H*)-one (118)

Reaction of **22** with 2-bromothiophene **85** according to general procedure A, followed by purification by flash column chromatography on silica gel (20% EtOAc/hexanes as eluant), gave **118** as a crystalline cream solid (61%); mp (EtOAc/*n*-pentane) 180–182 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 7.95 (d, *J* = 0.6 Hz, 1H), 7.90 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.85 (dd, *J* = 8.1, 0.5 Hz, 1H), 7.74 (dd, *J* = 3.7, 1.1 Hz, 1H), 7.71 (dd, *J* = 5.1, 1.1 Hz, 1H), 7.22 (dd, *J* = 5.1, 3.7 Hz, 1H), 5.44 (s, 2H). LRMS (APCI$^+$) calcd for $C_{12}H_9O_2S$ 217 (MH^+), found 217. Anal. ($C_{12}H_8O_2S$) C, H, N.

4.1.90. 5-(5-Acetylthiophen-2-yl)isobenzofuran-1(3*H*)-one (119)

2-Acetyl-5-bromothiophene **86** was reacted with **22** according to general procedure A, followed by flash column chromatography on silica gel (CH_2Cl_2 as eluant), to give **119** as a pale yellow solid (24%); mp (EtOAc/*n*-hexane) 193–196 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.08 (br s, 1H), 7.97–8.02 (m, 2H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.84 (d, *J* = 4.0 Hz, 1H), 5.46 (s, 2H), 2.57 (s, 3H). LRMS (APCI$^+$) calcd for $C_{14}H_{11}O_3S$ 259 (M+H), found 259.Anal. ($C_{14}H_{10}O_3S$) C, H, N.

4.1.91. 2-(5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)acetic acid (120)

5-Bromo-2-thiopheneacetic acid **87** was reacted with **22** according to general procedure A. The crude product was isolated by acidification of the basic aqueous layer with concd HCl upon

workup. ^{1}H NMR showed a mixture of two products; the desired tricylic compound and a product resulting from ring-opening of the lactone. This mixture was stirred with TFA to ring-close back to a single desired product. Removal of the TFA under reduced pressure afforded a residue which was dissolved in CH_2Cl_2 and then also removed under reduced pressure. This procedure was repeated twice, then the resulting solid triturated with Et_2O and collected by filtration to give **120** as a beige solid (75%); mp (Et_2O) 211–214 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.66 (br s, 1H), 7.91 (br s, 1H), 7.82–7.86 (m, 2H), 7.58 (d, J = 3.7 Hz, 1H), 7.03 (d, J = 3.8 Hz, 1H), 5.43 (s, 2H), 3.89 (s, 2H). HRMS (ESI$^+$) calcd for $C_{14}H_{10}NaO_4S$ 297.0192 (M+Na), found 297.0197.

4.1.92. Methyl 2-(5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)acetate (121)

Compound **120** (140 mg, 0.51 mmol) was dissolved in a mixture of Et_2O (4 mL) and MeOH (2 mL), then treated dropwise with 2 M trimethylsilyldiazomethane in Et_2O (0.38 mL, 0.77 mmol). After stirring for 1 h. at rt, AcOH was added dropwise to quench the reaction. All solvent was then removed under reduced pressure, the resulting residue dissolved in EtOAc (50 mL) and then washed with satd $NaHCO_3$ solution (3 × 50 mL), water (50 mL), brine (50 mL), dried (Na_2SO_4) and filtered. After removal of the solvent under reduced pressure the crude product was purified by flash column chromatography on silica gel (10% acetone/CH_2Cl_2 as eluant) to give **121** as a beige solid (87 mg, 59%); mp (Et_2O) 119–121 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 7.91 (br s, 1H), 7.82–7.86 (m, 2H), 7.60 (d, J = 3.7 Hz, 1H), 7.06 (d, J = 3.7 Hz, 1H), 5.43 (s, 2H), 4.01 (s, 2H), 3.67 (s, 3H). LRMS (APCI$^+$) calcd for $C_{15}H_{13}O_4S$ 289 (M+H), found 289. Anal. ($C_{15}H_{12}O_4S$) C, H, N.

4.1.93. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)imidazolidine-2,4-dione (122)

The title compound was prepared by reaction of **43** with hydantoin according to general procedure C to give **122** as a yellow solid (44%); mp (EtOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 10.20–11.50 (v br s, 2H), 7.96 (br s, 1H), 7.86–7.92 (m, 2H), 7.81 (d, J = 4.0 Hz, 1H), 7.65 (d, J = 4.0 Hz, 1H), 6.60 (s, 1H), 5.49 (s, 2H). Anal. ($C_{16}H_{10}N_2O_4S$) C, H, N.

4.1.94. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)-2-thioxothiazolidin-4-one (123)

Reaction of **43** with 2-thioxo-4-thiazolidinone according to general procedure D gave **123** as a dark orange solid (95%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 13.85 (br s, 1H), 8.10 (br s, 1H), 8.01 (dd, J = 8.1, 1.4 Hz, 1H), 7.87–7.95 (m, 3H), 7.76 (br d, J = 3.4 Hz, 1H), 5.46 (s, 2H). LRMS (APCI$^+$) calcd for $C_{16}H_{10}NO_3S_3$ 360 (MH$^+$), found 360. Anal. ($C_{16}H_9NO_3S_3$) C, H, N.

4.1.95. (*E,Z*)-5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)thiazolidine-2,4-dione (124)

Reaction of **43** with 2,4-thiazolidinedione according to general procedure D gave **124** as a bright orange solid (96%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.50 (br s, 1H), 8.03–8.88 (m, 2H), 7.98 (dd, J = 8.1, 1.5 Hz, 1H), 7.87–7.93 (m, 2H), 7.74 (d, J = 4.1 Hz, 1H), 5.46 (s, 2H). LRMS (APCI$^+$) calcd for $C_{16}H_{10}NO_4S_2$ 344 (MH$^+$), found 344. Anal. ($C_{16}H_9NO_4S_2$) C, H, N.

4.1.96. 5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (125)

Reaction of **43** with barbituric acid according to general procedure D gave **125** as a yellow-brown solid (100%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.31 (br s, 2H), 8.52 (s, 1H), 8.23 (d, J = 4.2 Hz, 1H), 8.15 (br s, 1H), 8.05 (dd, J = 8.1, 1.4 Hz, 1H), 7.95 (s, 1H), 7.93 (d, J = 3.6 Hz, 1H), 5.47 (s, 2H). Anal. ($C_{17}H_{10}N_2O_5S$) C, H, N.

4.1.97. 5-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (126)

Reaction of **43** with 4,6-dihydroxy-2-mercaptopyrimidine according to general procedure D gave **126** as a dark orange solid (100%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 12.42 (s, 2H), 8.55 (s, 1H), 8.29 (d, J = 4.4 Hz, 1H), 8.18 (d, J = 0.7 Hz, 1H), 8.07 (dd, J = 1.5, 8.0 Hz, 1H), 7.98 (d, J = 4.4 Hz, 1H), 7.94 (d, J = 8.0 Hz, 1H), 5.47 (s, 2H). Anal. ($C_{17}H_{10}N_2O_4S_2{\cdot}0.5H_2O$) C, H, N.

4.1.98. 1,3-Dimethyl-5-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (127)

Reaction of **43** with 1,3-dimethylbarbituric acid according to general procedure D gave **127** as a orange solid (99%); mp (AcOH) >300 °C. ^{1}H NMR (CF_3COOD) δ 9.06 (s, 1H), 8.10–8.17 (m, 4H), 7.83 (d, J = 4.2 Hz, 1H), 5.61 (s, 2H), 3.62 (s, 3H), 3.60 (s, 3H). LRMS (APCI$^+$) calcd for $C_{19}H_{15}N_2O_5S$ 383 (MH$^+$), found 383. Anal. ($C_{19}H_{14}N_2O_5S$) C, H, N.

4.1.99. (*E,Z*)-2-Imino-1-methyl-5-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)imidazolidin-4-one (128)

Reaction of **43** with creatine according to general procedure D gave **128** as a yellow-orange solid (95%); mp (AcOH) >300 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.91 (v br s, 1H), 7.94 (br s, 1H), 7.96 (br s, 1H), 7.84–7.91 (m, 2H), 7.67 (d, J = 3.9 Hz, 1H), 7.63 (d, J = 4.2 Hz, 1H), 6.61 (s, 1H), 5.44 (s, 2H), 3.21 (s, 3H). HRMS (FAB$^+$) calcd for $C_{17}H_{14}N_3O_3S$ 340.0756 (MH$^+$), found 340.07550. Anal. ($C_{17}H_{13}N_3O_3S{\cdot}0.75H_2O$) C, H, N.

4.1.100. 5-(5-(Oxazol-5-yl)thiophen-2-yl)isobenzofuran-1(3*H*)-one (129)

Compound **43** (100 mg, 0.41 mmol), tosylmethyl isocyanide (88 mg, 0.45 mmol) and K_2CO_3 (113 mg, 0.82 mmol) were suspended in MeOH (10 mL), stirred at rt for 5 min., then heated at reflux for 1 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and the resulting solution evaporated onto a small quantity of silica under reduced pressure. Purification was carried out by flash column chromatography on silica gel (50% EtOAc/hexanes as eluant) to give **129** as a pale yellow solid (93 mg, 80%); mp (Et_2O) 229–231 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.47 (s, 1H), 8.00 (s, 1H), 7.93 (dd, J = 8.0, 1.4 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.81 (d, J = 3.9 Hz, 1H), 7.63 (s, 1H), 7.57 (d, J = 3.9 Hz, 1H), 5.45 (s, 2H). LRMS (APCI$^+$) calcd for $C_{15}H_{10}NO_3S$ 284 (MH$^+$), found 284. Anal. ($C_{15}H_9NO_3S$) C, H, N.

4.1.101. (*E,Z*)-3-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)pyrrolidine-2,5-dione (130)

Compound **43** (100 mg, 0.41 mmol) was suspended in MeOH (5 mL) and triphenylphosphoranylidenesuccinimide **89** (147 mg, 0.41 mmol) (which was in turn prepared from succinimide **88** according to a literature procedure[39]) added. This mixture was heated at reflux for 1 h. Upon cooling, the resulting crude product was collected by filtration from the reaction mixture and purified by trituration with acetone. The title compound **130** was isolated as a yellow solid (83 mg, 62%); mp (acetone) >295 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 11.43 (s, 1H), 8.05 (s, 1H), 7.97 (dd, J = 8.1, 1.5 Hz, 1H), 7.90 (d, J = 8.1 Hz, 1H), 7.85 (d, J = 3.9 Hz, 1H), 7.64 (m, 2H), 5.46 (s, 2H), 3.55 (s, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{12}NO_4S$ 326 (MH$^+$), found 326. Anal. ($C_{17}H_{11}NO_4S$) C, H, N.

4.1.102. General procedure I: (*E,Z*)-1-benzyl-3-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)pyrrolidin-2-one (131)

Diethyl 1-(benzyl)-2-oxopyrrolidin-3-ylphosphonate[40] **90** (212 mg, 0.68 mmol) was dissolved in THF (6 mL), to which was added 18-crown-6 (902 mg, 3.41 mmol). This mixture was cooled

to -78 °C under N_2, then a 0.5 M solution of potassium bis(trimethylsilyl)amide in toluene (1.50 mL, 0.75 mmol) added dropwise. After 30 min, a solution of **43** (200 mg, 0.82 mmol) in THF (10 mL) was added and the solution allowed to warm to rt and stir for 18 h. NH_4Cl (satd, 50 mL) was added to the reaction mixture which was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic fractions were dried (Na_2SO_4), filtered, and the solvent removed under reduced pressure to yield a solid which was purified by flash column chromatography on silica gel (5% acetone/CH_2Cl_2 as eluant). The title compound **131** was isolated as a yellow solid (114 mg, 42%); mp (Et_2O) 205–209 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.01 (s, 1H), 7.93 (dd, J = 8.1, 1.4 Hz, 1H), 7.88 (d, J = 7.0 Hz, 1H), 7.82 (d, J = 3.9 Hz, 1H), 7.52 (d, J = 4.0 Hz, 1H), 7.46 (t, J = 2.8 Hz, 1H), 7.33–7.39(m, 2H), 7.25–7.32 (m, 3H), 5.44 (s, 2H), 4.56 (s, 2H), 3.45 (t, J = 6.3 Hz, 2H), 2.96–3.02 (m, 2H). LRMS (APCI$^+$) calcd for $C_{24}H_{20}NO_3S$ 402 (MH^+), found 402. Anal. ($C_{24}H_{19}NO_3S$) C, H, N.

4.1.103. (*E,Z*)-1-(4-Methoxybenzyl)-3-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)pyrrolidin-2-one (132)

Diethyl 1-(4-methoxybenzyl)-2-oxopyrrolidin-3-ylphosphonate **91** (prepared by adapting a literature procedure[40]) was reacted with **43** according to general procedure I. Purification was carried out by flash column chromatography on silica gel (5% acetone/CH_2Cl_2 as eluant) to give **132** as a yellow solid (17%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.00 (d, J = 0.6 Hz, 1H), 7.93 (dd, J = 8.1, 1.5 Hz, 1H), 7.87 (d, J = 8.1 Hz, 1H), 7.82 (d, J = 3.9 Hz, 1H), 7.51 (d, J = 4.0 Hz, 1H), 7.44 (t, J = 2.7 Hz, 1H), 7.21 (dt, J = 8.7, 2.4 Hz, 2H), 6.92 (dt, J = 8.7, 2.5 Hz, 2H), 5.44 (s, 2H), 4.48 (s, 2H), 3.74 (s, 3H), 3.41 (t, J = 6.2 Hz, 2H), 2.97 (m, 2H). HRMS (FAB$^+$) calcd for $C_{25}H_{22}NO_4S$ 432.1270 (MH^+), found 432.1269.

4.1.104. General procedure J: (*E,Z*)-3-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)pyrrolidin-2-one (133)

Compound **132** (71 mg, 0.17 mmol) was dissolved in TFA (5 mL), to which was added anisole (5 drops). This mixture was heated at reflux for 15 h. then the solvent removed under reduced pressure. The resulting residue was dissolved in 5% MeOH/CH_2Cl_2 (50 mL) and washed with satd $NaHCO_3$ (50 mL). The solution was dried over Na_2SO_4, filtered, and evaporated onto silica prior to purification by flash column chromatography (5% MeOH/CH_2Cl_2 as eluant) to afford **133** as a yellow solid (39 mg, 71%), mp (Et_2O/CH_2Cl_2) 224–227 °C. ^{1}H NMR [$(CD_3)_2SO$] δ 8.17 (br s, 1H), 3.01 (br s, 1H), 7.93 (dd, J = 8.1, 1.5 Hz, 1H), 7.88 (d, J = 7.9 Hz, 1H), 7.81 (d, J = 3.9 Hz, 1H), 7.48 (d, J = 4.0 Hz, 1H), 7.34 (t, J = 2.8 Hz, 1H), 5.45 (s, 2H), 3.44 (t, J = 6.3 Hz, 2H), 3.01 (td, J = 6.4, 2.8 Hz, 2H). LRMS (APCI$^+$) calcd for $C_{17}H_{14}NO_3S$ 312 (MH^+), found 312. Anal. ($C_{17}H_{13}NO_3S$) C, H, N.

4.1.105. Diethyl 1-(4-methoxybenzyl)-2-oxopiperidin-3-ylphosphonate (92)

The title compound **92** was prepared by adapting a literature procedure[40] and was isolated as a pale yellow oil (27%). ^{1}H NMR [$(CD_3)_2SO$] δ 7.20 (d, J = 8.6 Hz, 2H), 6.84 (d, J = 8.7 Hz, 2H), 4.63 (d, J = 14.6 Hz, 1H), 4.47 (d, J = 14.6 Hz, 1H), 4.12–4.29 (m, 4H), 3.79 (s, 3H), 3.21–3.29 (m, 1H), 3.12–3.20 (m, 1H), 3.08 (t, J = 6.5 Hz, 0.5H), 3.02 (t, J = 6.5 Hz, 0.5H), 2.15–2.27 (m, 1H), 2.01–2.14 (m, 2H), 1.34 (q, J = 7.0 Hz, 6H). LRMS (APCI$^+$) calcd for $C_{17}H_{26}NO_5P$ 356 (MH^+), found 356.

4.1.106. (*E,Z*)-1-(4-Methoxybenzyl)-3-((5-(1-oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)piperidin-2-one (93)

Compound **92** was reacted with **43** according to general procedure I. Purification was carried out by flash column chromatography on silica gel (5% acetone/CH_2Cl_2 as eluant). The title compound **93** was isolated as a yellow solid (29%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.02 (s, 1H), 7.95 (dd, J = 8.1, 1.4 Hz, 1H), 7.88 (d, J = 8.2 Hz, 1H), 7.86 (br s, 1H), 7.82 (d, J = 3.9 Hz, 1H), 7.51 (d, J = 4.0 Hz, 1H), 7.22 (d, J = 8.7 Hz, 2H), 6.90 (d, J = 8.7 Hz, 2H), 5.44 (s, 2H), 4.57 (s, 2 H), 3.74 (s, 3H), 3.34 (br t, J = 5.7 Hz, 2H), 2.84 (br t, J = 5.8 Hz, 2H), 1.86 (pentet, J = 6.0 Hz, 2H). LRMS (APCI$^+$) calcd for $C_{26}H_{23}NO_4S$ 446 (MH^+), found 446.

4.1.107. (*E,Z*)-3-((5-(1-Oxo-1,3-dihydroisobenzofuran-5-yl)thiophen-2-yl)methylene)piperidin-2-one (134)

Compound **93** was deprotected according to general procedure J to give **134** as a yellow solid (85%). ^{1}H NMR [$(CD_3)_2SO$] δ 8.02 (br s, 1H), 7.95 (dd, J = 8.1, 1.5 Hz, 1H), 7.88 (d, J = 8.0 Hz, 1H), 7.83 (br s, 1H), 7.81 (d, J = 4.0 Hz, 1H), 7.76 (br t, J = 2.0 Hz, 1H), 7.50 (d, J = 4.0 Hz, 1H), 5.44 (s, 2H), 3.22–3.28 (m, 2H), 2.78–2.84 (m, 2H), 1.85 (pentet, J = 6.0 Hz, 2 H). HRMS (FAB$^+$) calcd for $C_{18}H_{16}NO_3S$ 326.0851 (MH^+), found 326.0846.

4.2. Biology

4.2.1. Inhibition of perforin-mediated lysis of sheep red blood cells (SRBC)

As reported previously[29] compound **2** was identified from screening a commercial library of ~100,000 compounds for the ability to reproducibly inhibit perforin-mediated lysis of SRBC at a compound concentration of 100 μM.

4.2.2. Inhibition of perforin-mediated lysis of Jurkat cells

The ability of the compounds to inhibit the lysis of nucleated (Jurkat T lymphoma) cells in the presence of 0.1% BSA, as measured by release of ^{51}Cr was measured. Jurkat target cells were labelled by incubation in medium with 100 μCi ^{51}Cr for one hour. The cells were then washed three times to remove unincorporated isotope and re-suspended at 1 × 10^5 cells per mL in RPMI buffer supplemented with 0.1% BSA. Each test compound was pre-incubated to concentrations of 20, 10, 5, 2.5 and 1.25 μM with recombinant perforin for 30 min with DMSO as a negative control. ^{51}Cr labeled Jurkat cells were then added and cells were incubated at 37 °C for 4 h. The supernatant was collected and assessed for its radioactive content on a gamma counter (Wallac Wizard 1470 automatic gamma counter). Each data point was performed in triplicate and an IC_{50} was calculated from the range of concentrations described to above. Compounds with an IC_{50} <1 μM were titrated down to lower concentrations in the same manner as above, to determine an accurate IC_{50}.

4.2.3. Inhibition of PLY-mediated lysis of Jurkat cells

In order to determine whether perforin inhibitor **2** was also able to block the lytic function of PLY, it was tested at a concentration of 20 μM in the presence PLY instead of perforin with ^{51}Cr labeled Jurkat target cells as described above.

4.2.4. KHYG-1 cytotoxicity assay

KHYG-1 cells were washed and resuspended in RPMI + 0.1% BSA at 4 × 10^5 cells/mL and 50 μL of KHYG-1 cells were dispensed to each well of a 96-well V-bottom plate. RPMI (50 μL) + 0.1% BSA or 10% (final concentration) of serum was added to each well then test compounds were added to KHYG-1 cells at various concentrations up to 20 μM and incubated at rt for 20 minutes. 1 × 10^6 K562 target cells were labelled with 75 μCi ^{51}Cr in 200 μL RPMI for 90 min at 37 °C, cells were washed as described above and resuspended in 5 ml RPMI + 0.1% BSA. 50 μL of ^{51}Cr labelled K562 leukemia target cells were added to each well of the KHYG-1 plate (Effector:Target 2:1) and incubated at 37 °C for 4 h. ^{51}Cr release was assayed using a Skatron Harvesting Press and radioactivity estimated on a Wallac Wizard 1470 Automatic Gamma counter

(Turku, Finland). The percentage of specific cytotoxicity was calculated by the formula:

$$\%\text{specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

and expressed as the mean of triplicate assays ± standard error of the mean.

4.2.5. Toxicity to KHYG-1 NK cells

The toxicity assay was carried out in exactly the same manner as the killing assay above, but instead of adding the labelled K562 target cells, 100 μL of RPMI 0.1% BSA was added. Cells were incubated for 4 h at 37 °C then washed ×3 in RPMI + 0.1% BSA. Cells were then resuspended in 200 μL of complete medium and incubated for 18–24 h at 37 °C. Trypan blue was added to each well and viable (clear) cells and total (clear + blue) cells were counted and the percentage of viable cells was calculated compared to DMSO treated cell control (% viability).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.011.

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