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Graphical Abstract

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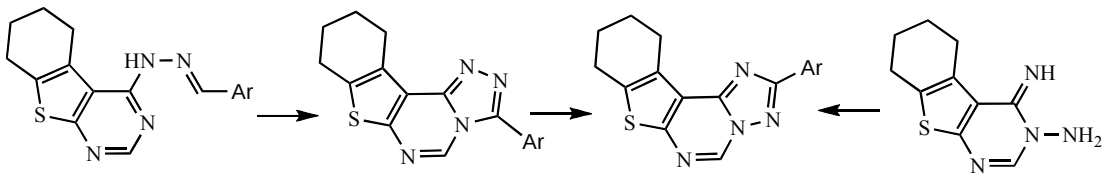

Simple oxidation of pyrimidinylhydrazones to triazolopyrimidines and their inhibition of Shiga toxin trafficking.

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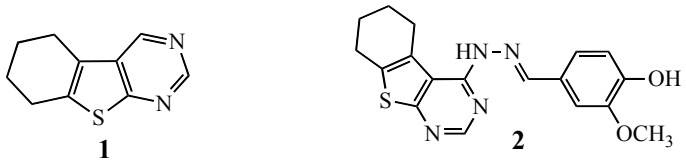
Abstract

The oxidative cyclisation of a range of benzothieno[2,3-d]pyrimidine hydrazones (**7a-e**) to the 1,2,4-triazolo[4,3-c]pyrimidines (**8a-e**) catalysed by lithium iodide or to the 1,2,4-triazolo[1,5-*c*]pyrimidines (**10a-e**) with sodium carbonate is presented. A complementary synthesis of the 1,2,4-triazolo[1,5-*c*]pyrimidines (**10a-e**) starting from the amino imine **11** is also reported. The effect of these compounds on Shiga toxin (STx) trafficking in HeLa cells and comparison to the previously reported Exo2 is also detailed

Keywords Pyrimidyl hydrazone, triazolopyrimidine, lithium iodide, DMSO, sodium carbonate, Dimroth rearrangement, Exo2, Shiga toxin

1. Introduction

Compounds containing the 5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidine (**1**) nucleus have found many applications as adenosine mimics [1], analgesics [2], anticancer [3], antiviral agents [4], and as inhibitors of kinases [5] and human epidermal growth factor [6].



1 **2**

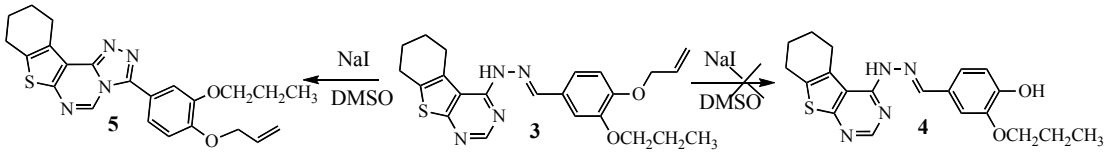
*Fig.1. The 5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidine core (**1**) and Exo2 (**2**)*

Exo2 (**2**) is a small molecule inhibitor of exocytosis that functions via the disruption of membrane trafficking [7]. This action also perturbs retrograde trafficking and as such, it has been used as a tool to help dissect the entry pathway of lipid binding bacterial toxins in mammalian cells [8,9]. Indeed, Exo2 has been shown to have a significant protective effect on HeLa cells against the protein synthesis inhibiting Shiga toxin (STx). Its effects on organelle morphology have suggested that its cellular target is involved in early endosome delivery to the TGN and/or TGN access to the Golgi stack [9]. By blocking delivery into the Golgi, Shiga toxin cannot reach the endoplasmic reticulum from where it normally translocates a membrane to reach its ribosome substrates in the cytosol.

This paper details our findings from the unexpected oxidative cyclisation of pyrimidyl hydrazones to give triazolopyrimidines, and includes a preliminary investigation on the ability of these new analogues of Exo2 to protect HeLa cells against a challenge by Shiga toxin.

2. Chemistry

During work on the synthesis of derivatives of Exo2 to discern structure activity relationships we required the hydrazone phenol (**4**). We had planned a simple deallylation of the ether (**3**) but concerned by the possible interference of the sulphur with palladium catalysed deallylation, we opted to use sodium iodide in dimethyl sulphoxide [10]. Instead of the expected deallylation a new compound was isolated

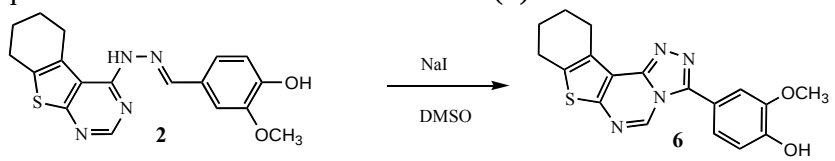


Scheme 1. Oxidative cyclisation during attempted deprotection of allyloxy ether

which still contained both the allyl and *n*-propyl ethers but lacked the NH and imine hydrogen of the hydrazone in ^{1}Hnmr and had 2 mass units less than the starting material. This was identified as the 1,2,4-triazolo[4,3-c]pyrimidine (**5**) with the aid of correlation spectroscopy which showed NOE interactions between the pyrimidyl CH and the phenyl CHs ortho to the hydrazone bond.

The many literature reports on the synthesis of the triazolopyrimidine fused ring system [11] either start form reaction of the pyrimidine hydrazine with an acid derivative (orthoformate [12] or activated acids [13]) or via oxidative cyclisation of the hydrazone with reagents like NBS [14], $Pb(OAc)_4$ [15], $FeCl_3$ [16], or iodobenzene diacetate [17].

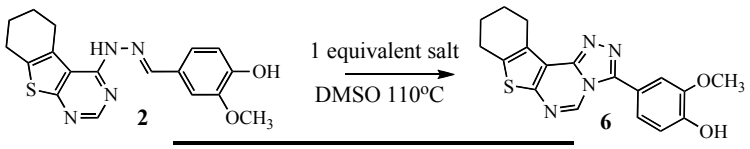
On treatment of Exo2 (**2**) with sodium iodide and DMSO we were pleased to obtain a quantitative conversion to the triazole (**6**).



Scheme 2. Sodium iodide catalysed oxidative cyclisation of Exo2

We examine other salts as possible additives. As can be seen from Table 1, there is no measurable reaction at room temperature and the reaction was best performed at 110°C. Without additive, the oxidative cyclisation in DMSO is slow with low conversion.

Table 1. Influence of the salt on the oxidative cyclisation of Exo2

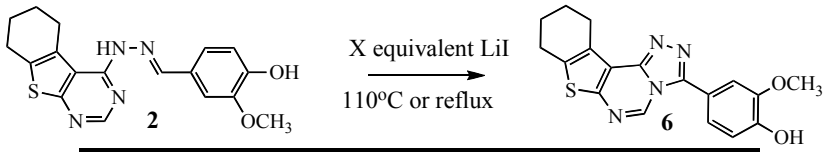


salt	time	**6**[a]
none	54hr	25%
NaI	24hr	>99%
NaBr	48hr	31% [b]
NaCl	48hr	24% [b]
Na_2SO_4	48hr	24%
KI	48hr	>99%
LiI	18hr	>99%
NMe_4I	48hr	>99%

*[a] conversion determined by NMR - [b] **2** and **6** are identified in the mixture, but also decomposition of the starting material.*

Other halide salts like sodium bromide were far less efficient than the iodide and sodium chloride was similar to the uncatalysed reaction in DMSO with a slow cyclisation and gradual decomposition at elongated reaction times. The other iodide salts tested all showed excellent ability for the cyclisation with lithium iodide being the most effective. The addition of excess lithium iodide does not notably enhance the rate, whereas the use of sub-molar quantities has a rate limiting effect.
Table 2 details the effect of solvent choice on the oxidative cyclisation reaction of Exo2 (**2**) catalysed by lithium iodide. The most favourable conditions use DMSO or DMF with little reactivity seen in other common solvents though in several cases this is more likely due to the low solubility of the starting material and lower reaction temperatures.

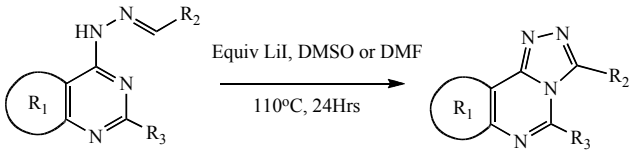
Table 2. Influence of the solvent on LiI catalysed oxidative cyclisation of Exo2



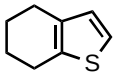
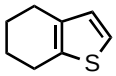
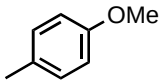
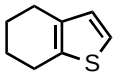
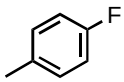
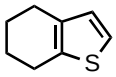
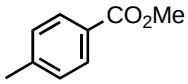
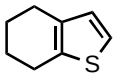
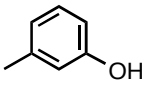
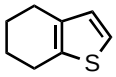
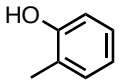
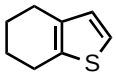
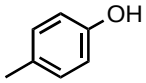
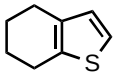
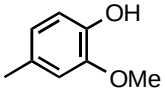
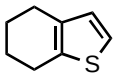
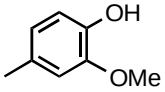
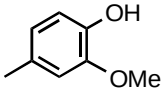
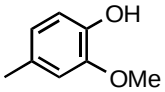
Solvent	LiI (equiv.)	Time	**2**[a]	**6**[a]
DMF	1	28hr	-	78%[b]
DMF	0	48hr	-	69%[b]
DMSO	1	24hr	-	>99%
DMSO	0	54hr	75%	25%
MeOH	1	48hr	>99%	-
EtOAc	1	48hr	83%	17%
acetone	1	48hr	>99%	-
ACN	1	48hr	>95%	<5%
ACN	0	48hr	>99%[c]	+
NO_2CH_3	1	48hr	>99%	-

[a] ratio determined by NMR - [b] isolated yield - [c] precipitate collected and NMR recorded in DMSO(>80% recovery)

Table 3 shows the range of pyrimidinylhydrazones **7a-e** that can be cyclised to the 1,2,4-triazolo[4,3-c]pyrimidines (**8a-e**) using lithium iodide in DMSO but the most convenient approach is to use lithium iodide in DMF. The triazole can simply be filtered off on cooling. The reported yields are unoptimised. The products are characterised by loss of the NH and imine hydrogen and the shift of the pyrimidine CH in ^{1}H nmr. The ^{1}H nmr and ^{13}C nmr of the hydrazone (~8.3ppm/152ppm in the starting hydrazone) shift to around 9.1ppm [22] and 135ppm respectively in the cyclised products (**8a-j**). Spectra and physical properties also match the known derivatives **8a** and **8b** [18]. Additionally, we were fortunate to obtain suitable crystals for X-ray diffraction of **8b** which confirmed the cyclised structure and the substitution pattern (Figure 2a).
Table 3. Oxidative cyclisation of pyridyl- and pyrimidyl- hydrazones to 1,2,4-triazolo[4,3-a]pyridines and 1,2,4-triazolo[4,3-a]pyrimidines



7a-j **8a-j**

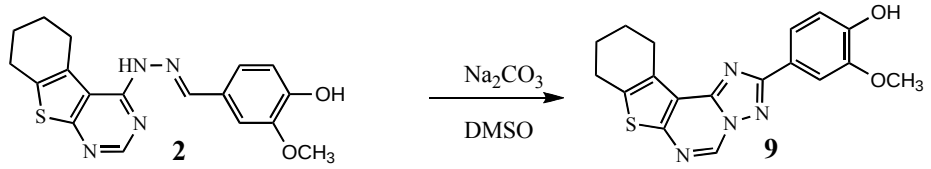
	R_1	R_2	R_3	X	Product	Conversion (DMSO) [a]	Yield (DMF) [b]
7a			H	N	**8a**	-	76%
7b			H	N	**8b**	-	64%
7c			H	N	**8c**	55% [c]	-
7d			H	N	**8d**	>80%	-
7e			H	N	**8e**	-	24%
7f			H	N	**8f**	-	52%
7g			H	N	**8g**	-	65%
2			H	N	**6**	>99%	78%
7h			CH_3	N	**8h**	>80%	-
7i			H	N	**8i**	32% [c]	-
7j	H-, H-		H	C	**8j**	>99%	-

[a] Conversion determined by NMR - [b] Isolated yields - [c] further heating leads to decomposition of the reaction mixture.

We originally suspected that I_2 was the reagent responsible for the cyclisation and that DMSO was the auxiliary oxidant generating the I_2 from iodide [19]. I_2 could act in an analogous manner to the reaction catalysed by NBS [14] and there is literature precedent for similar I_2 catalysed cyclisations of diaiminobenzenes and aldehydes to benzimidazoles [20]. However, running the reaction under nitrogen does not yield the cyclisation, with or without lithium iodide highlighting the requirement for oxygen. This was also corroborated by the discovery that the cyclisation of Exo2 (**2**) proceeds in DMF alone to give **6** with out salt addition and that reaction of hydrazone **2** with iodine in DMSO does not give the expected cyclised product **6** (*vida infra*). This lead us to reassess our hypothesis and that may be oxygen and small amounts of base (DMF is known to breakdown on heating to carbon monoxide and dimethylamine) were responsible for the cyclisation with the role of the lithium iodide unclear. Other common amide solvents like dimethyl acetamide and N-methyl pyrrolidinone which are not prone to the same degradation as DMF, were not so effective in the additive

free cyclisation of Exo2 (**2**). The limitation of the additive free cyclisation reaction in DMF is that it is only practical for hydrazones with an activating hydroxy group on the aromatic ring with little or no cyclised material observed for reaction of the fluoro **7c** or ester **7d** substituted hydrazones. These hydrazones require the presence of lithium iodide for efficient cyclisation.

Returning to the effect of salts in DMSO, we next investigated basic salts. Heating Exo2 (**2**) in DMSO with sodium carbonate gave a new compound the isomeric triazole (**9**). The reaction also proceeds with sodium acetate but is not so effective. The pyrimidine CH of the 1,2,4-triazolo[1,5-*c*]pyrimidine now shifts further down field to 9.54ppm [12b], but the associated ^{13}C signal still resonates around 135ppm. No NOE effect could be measured between the pyrimidyl CH and the ortho protons of the hydrazone in accord with the different orientation of the phenyl group of the isomerised triazole. The solid state structure of **9** from Xray crystallography is shown in Figure 2b confirming the substitution pattern of the triazole ring.

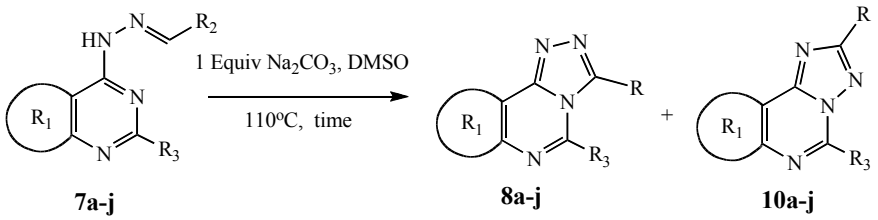


Scheme 3. *Oxidative cylisation and rearrangement of Exo2 with sodium carbonate*

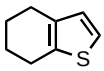
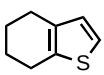
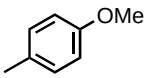
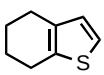
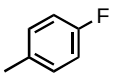
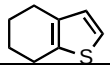
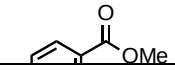
Sodium carbonate has catalysed both the oxidative cyclisation and Dimroth [21] type rearrangement of (**2**) to yield the 1,2,4-triazolo[1,5-*c*]pyrimidine (**9**). We presume that the 1,2,4-triazolo[4,3-*c*]pyrimidine **6** is an intermediate which then undergoes the Dimroth type rearrangement. Small quantities of the triazole **6** can be observed by ^{1}H nmr and thin layer chromatography as the reaction proceeds and the rearrangement of the triazole **6** to its isomeric1,2,4-triazolo[1,5-*c*] pyrimidine **9** is in accord with published work under similar basic conditions [12, 22].

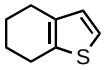
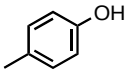
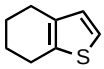
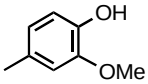
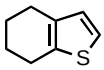
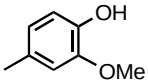
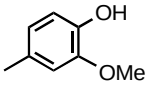
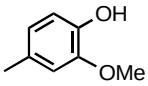
The application of these basic conditions to the range of hydrazones to catalyse the oxidative cyclisation and rearrangement was followed by ^{1}H nmr and is reported in Table 4.

Table 4. Oxidative cyclisation of pyridyl- and pyrimidyl- hydrazones to 1,2,4-triazolo[1,5-c]pyridines **8a-j** and 1,2,4-triazolo[1,5-c]pyrimidines **10a-j**



7a-j **8a-j** **10a-j**

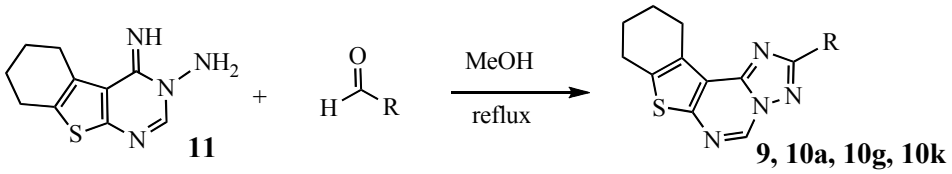
	R_1	R_2	R_3	X	Time	Product	Ratio % [a]
						7a	50%
7a			H	N	2d [b]	**8a**	50%
						10a	-
						7b	20%
7b			H	N	2d [b]	**8b**	-
						10b	80%
						7c	30%
7c			H	N	5d [b]	**8c**	5%
						10c	65%
						7d	-
7d			H	N	24hr [b]	**8d**	-

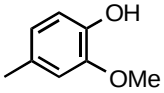
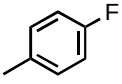
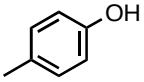
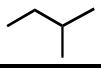
						10d	>90%
						7g	-
7g			H	N	4d	**8g**	-
						10g	>99%
						2	-
2			H	N	2d	**6**	-
						9	>99%
						7h	-
7h			CH_3	N	3d	**8h**	-
						10h	>99%
						7i	45%
7i			H	N	4d [b]	**8i**	10%
						10i	45%
						7j	45%
7j	H-, H-		H	C	4d [b]	**8j**	10%
						10j	45%

[a] ratio determined by NMR - [b] further heating leads to decomposition

In general, only compounds containing a 4-hydroxy group and a thiopyrimidine ring underwent efficient transformation to the1,2,4-triazolo[1,5-*c*]pyrimidines (**9**, **10g**, and **10h**) but this is not so clear cut as the ester substituted hydazone **7d** is a good substrate but the hydroxy containing phenylpyrimidine **7i** reacts slowly. Following these reactions by ^{1}H nmr, the initial cyclisation catalysed by sodium carbonate seems to be inefficient in some of these substrates and other reaction pathways start to predominate rather than the oxidative cyclisation and isomerisation to the 1,2,4-triazolo[1,5-*c*]pyrimidine product.
Investigation of the related reaction of amino imine **11** and aldehydes also furnishes the 1,2,4-triazolo[1,5-*c*]pyrimidines (**9**, **10a**, **10c**, **10g**, and **10k**) shown in Table 5.

Table 5. Synthesis of 1,2,4-triazolo[1,5-c]pyrimidines **2**, **10a, 10g and 10k** starting from the amino imine **11**.

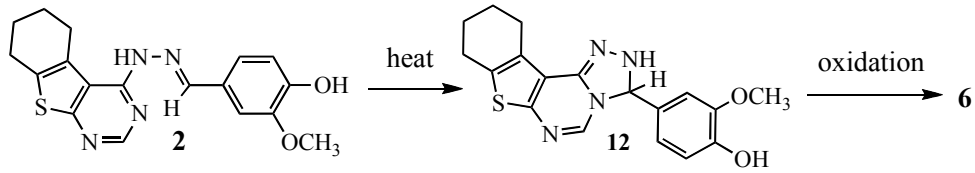


product	**R**	Yield
9		65%
10a		66%
10c		??
10g		34%
10k		70%

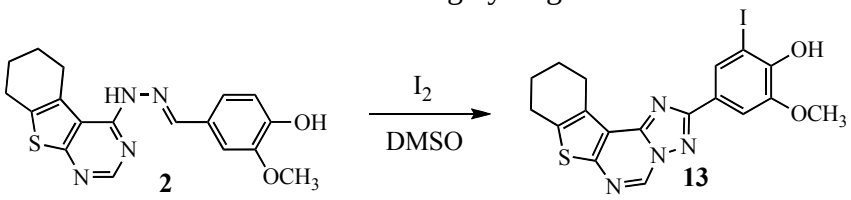
Similar cyclisations have been reported but required acetic acid catalysis [23].
The most practical procedure was simply to reflux equimolar amounts of the amino imine **11** and the required aldehyde in methanol. Salt addition is not required and

unlike the sodium carbonate oxidative cyclisation above, there are no constraints on the starting aldehyde nor the requirement to start from the hydrazone. Simple filtration gives good yields for both electron rich and electron deficient benzaldehydes and aliphatic aldehydes (Table 5).
This reaction is very similar to the cyclisation reaction of **2** to **9** involving the cyclisation of an aldehyde derived intermediate and arial oxidation.

The arial oxidation probably goes via a diamino acetal **12** as shown in scheme 4. There are several other examples of the arial oxidation of other dinitrogen containing heterocycles [3a, 24] and the benzylic nature and hydroxy substitution would all aid a radical oxidation. Adding a catalytic amount of AIBN to a salt free DMSO solution of hydrzaone **2** gave an increase in the amount of oxidative cyclisation product **6** compared to DMSO strengthening the idea that the reaction goes by a radical oxidation.



Scheme 4. *Possible diamino acetal intermediate in the oxidative cyclisation of Exo2*
We finally looked at the reaction of **2** with an equivalent of iodine in DMSO which does lead to a rapid oxidative cyclisation but to the isomeric 1,2,4-triazolo[1,5-*c*]pyrimidine with concomitant iodinisation to give isomer **15** based on the coupling constant between the two remaining hydrogens on the electron rich phenolic ring.



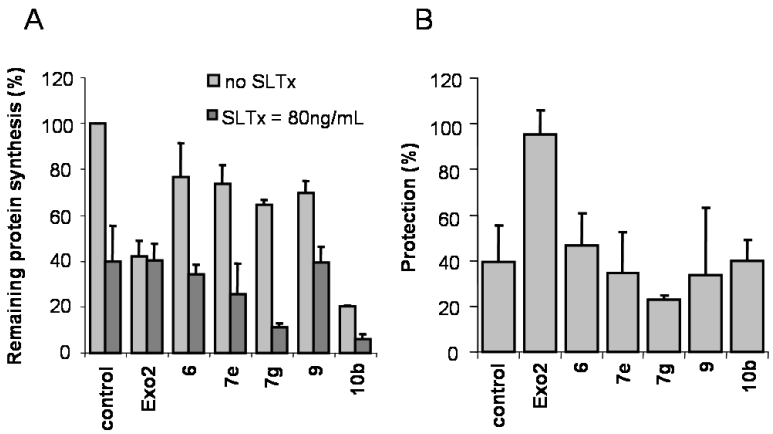
Scheme 5. *Iodine catalysed oxidation and rearrangement of Exo2*
Application of these conditions to the hydrazone **7a** also gives the oxidative cyclisation and rearrangement to the triazole isomer **10a** without the iodination of the less reactive phenyl ring. The generation of hydrogen iodide must catalyse the Dimroth type rearrangement of the intermediate 1,2,4-triazolo[4,3-*c*]pyrimidine to the isomeric 1,2,4-triazolo[1,5-*c*]pyrimidine ring system in a similar manner to other acid catalysed Dimroth rearrangement [12, 22, 23a, 25]. This again would indicate that the lithium iodide catalysed cyclisation does not involve the direct oxidation via elemental iodine. Iodide may reduce a peroxide intermediate from arial oxidation of the diamine precursor **12** or there may be a role for small amounts of iodine to mediate in the radical oxidation as has recently been described for the addition-fragmentation chain transfer processes in living radical polymer synthesis [26].

3. Inhibition of Shiga toxin trafficking

The new triazoles represent cyclised Exo2 derivatives that lack a potentially hydrolysable hydrazone linkage between the thienopyrimidine core and the phenyl substituent. Additionally, the different triazole isomers allow the presentation of the phenyl ring and its substituents in different orientations to optimise structure activity relationships accessing three alternative compounds from the one hydrazone starting material. The 1,2,4-triazolo[4,3-*c*]pyrimidines analogues **6**, **8e**, **8g** and the 1,2,4-triazolo[1,5-*c*]pyrimidines **9** and **10b** (the other compounds were either too insoluble

or showed no meaningful activity in this assay) were tested in parallel for their inherent toxicity towards protein synthesis and also their ability to confer a protective effect on HeLa cells from a STx challenge in comparison to Exo2, by modifying a previously published procedure [9].

Figure 2. Biological activity of the triazolopyrimidines



Graph A – protein synthesis on treatment with compound (light grey bars) and on treatment with compound and STx (dark grey bars) normalised to carrier DMSO
Graph B – protective effect as described by the ratio of protein synthesis in the presence of the test compound with and without STx challenge

To summarise, the compounds were first assessed for their inherent cytotoxicity, based on the level of protein synthesis remaining in chemical-treated HeLa cells when normalised against protein synthesis levels in cell treated with the DMSO carrier alone (Figure 2 Graph A, light grey bars). The procedure was replicated but this time, the cells were challenged for 1 hour with a dose of Shiga toxin previously determined to promote a 60% drop in protein synthesis of the control, and the level of remaining protein synthesis measured (Figure 2 Graph A, dark gray bars). Most of the triazoles were substantially less toxic than Exo2 in this regard, although **10b** inhibited protein synthesis significantly more than Exo2. When challenged with toxin, Exo2-treated cells almost completely retain their level of protein synthesis under these conditions (Graph A, light grey dark grey bars). The protective effect (Figure 2 Graph B) is quantified by comparing protein synthesis from exposure to just the compound with protein synthesis after treatment with the compound and a toxin challenge, and takes into account any effect of the compound alone. All of the triazoles examined were less effective in this comparison than Exo2. The loss of an NH hydrogen bond donating group of the hydrazone linkage and the tethering of the substituted phenyl group into a triazole ring may significantly alter the orientation of the phenyl ring from that adopted as a hydrazone in Exo2 and any advantages from using non-hydrolysable analogues is not revealed under these conditions.

4. Conclusions.

In conclusion, mild and simple conditions have been discovered to produce the oxidative cyclisation of pyrimidine hydrazones to 1,2,4-triazolo[4,3-*c*]pyrimidines by heating in DMSO or DMF. Lithium iodide is required for efficient cyclisation of starting hydrazones that lack an activating 4-hydroxyphenyl substituent and no further

isomerisation takes place under these conditions. These triazoles undergo rearrangement to the isomeric 1,2,4-triazolo[1,5-*c*]pyrimidines using sodium carbonate in DMSO or can be furnished directly from oxidative cyclisation and rearrangement of some appropriately substituted starting hydrazones. A more general complementary procedure as exemplified by the synthesis of the 1,2,4-triazolo[1,5-*c*]pyrimidines **9**, **10a**, **10c**, **10g**, and **10k** starting from the amino imine **11** was also realised.

Although we have been unable to elucidate a mechanism, the reactions discussed above allow routes to specific triazolopyrimidine isomers from aldehyde precursors by simple arial oxidation.

These triazoles were tested in a toxin challenge assay in comparison to Exo2. Although most showed a reduced level of inhibition of protein synthesis than Exo2, they were less effective in giving a protective effect against Shiga toxin than Exo2 in this assay.

Whether they protect against alternative toxins that follow the retrograde pathway was not investigated here. Nevertheless, the novel compounds described in this report may provide a valuable platform for future studies.

5.1 Experimental

All reagents and solvents were purchased from Lancaster and Aldrich and used without further purification. The hydrazone starting materials were synthesised as detailed elsewhere [9]. NMR spectra were recorded on a DPX-400 spectrometer at room temperature (298K). The spectra were recorded in parts per million (ppm) and referenced using residual protio solvents relative to trimethylsilane standard (δ_H = 0ppm). 2D COSY, HMQC, HMBC and NOSEYspectra were used to aid with peak assignments. ESI mass spectra were obtained using a Bruker Esquire 2000 mass spectrometer coupled with an Agilent 1100 HPLC (without a column) as the delivery system. Accurate mass spectra were obtained using a Bruker micro-TOF ESI attached to a time of flight (TOF) analyzer. Infrared spectra were gathered using a Perkin-Elmer Paragon 1000 FTIR spectrometer. CHN elemental analyses were carried out by Warwick Analytical Services. Thin Layer Chromatography used in monitoring reaction progress were performed using silica layer (0.25mm) coated alumina plates. Weights were recorded on a balance to 4 decimal places.

*5.1.1 General procedure for lithium iodide catalysed oxidative cyclisation of pyrimidyl hydrazones to give the 1,2,4-triazolo[4,3-c]pyrimidines (**6**,**7a-e**)*

The hydrazone (5mmole) was dissolved in reagent grade DMF (15ml) and one equivalent of lithium iodide was added (1 equivalent, 0.7g). The mixture was stirred and heated at 110°C under an air atmosphere. After the specified time (24 or 48hrs), the reaction was allowed to cool and the precipitate isolated by filtration and washed with a little methanol and then diethyl ether and dried under high vacuum.

5.1.1.1 3-(4-Hydroxy-3-methoxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **6**

^{1}H NMR (400 MHz, DMSO) δ: 1.93 (m, 4H, 2 CH_2), 3.12 (m, 2H, CH_2), 3.35 (m, 2H, CH_2), 3.90 (s, 3H, OCH_3), 7.02 (d, J = 8.0Hz, 1H, CH), 7.39 (dd, J_1 = 8.0Hz, J_2 = 2.0Hz, 1H, CH), 7.50 (d, J = 2.0Hz, 1H, CH), 9.20 (s, 1H, CH), 9.69 (brs, 1H, OH).

^{13}C NMR (100 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 24.8 (CH_2), 25.2 (CH_2), 55.8 (OCH_3), 112.4 (CH), 115.9 (CH), 116.6 (C), 117.8 (C), 121.8 (CH), 129.2 (C), 134.5

(CH), 137.7 (C), 146.0 (C), 146.1 (C), 148.0 (C), 148.7 (C), 148.8 (C). ES-MS *m*/*z* 353.1 (MH^+). HRMS 353.1067, found 353.1068. Anal. Calcd for $C_{18}H_{16}N_4O_2S$: C 61.35, H 4.68, N 15.90; found: C 60.35, H 4.51, N 15.57.

5.1.1.2 3-Phenyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8a**

^{1}H NMR (400 MHz, DMSO) δ: 1.94 (m, 4H, 2 CH_2), 2.93 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 7.66 (m, 3H, 3 CH), 8.01 (m, 2H, 2 CH), 9.22 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 24.8 (CH_2), 25.2 (CH_2), 117.8 (C), 125.9 (C), 128.6 (4 CH), 128.6 (C), 129.2 (C), 129.3 (CH), 130.4 (CH), 137.9 (C), 145.8 (C), 146.3 (C). ES-MS *m*/*z* 307.1 (MH^+). HRMS 307.1012, found 307.1015.

5.1.1.3 3-(4-methoxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8b**

^{1}H NMR (400 MHz, DMSO) δ: 1.93 (m, 4H, 2 CH_2), 2.93 (m, 2H, CH_2), 3.12 (m, 2H, CH_2), 3.89 (s, 3H, OCH_3), 7.19 (d, *J* = 8.8Hz, 2H, CH), 7.93 (d, *J* = 8.8Hz, 2H, CH), 9.16 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 24.8 (CH_2), 25.1 (CH_2), 55.4 (OCH_3), 114.7 (CH), 117.8 (C), 118.1 (C), 129.2 (C), 130.2 (CH), 134.3 (CH), 137.8 (C), 145.7 (C), 146.1 (C), 148.8 (C), 160.8 (C). ES-MS *m*/*z* 337.1 (MH^+). HRMS 337.1118, found 337.1108.

5.1.1.4 3-(3-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8e**

^{1}H NMR (300 MHz, DMSO) δ: 1.94 (m, 4H, 2 CH_2), 2.94 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 7.03 (d, *J* = 7.6Hz, 1H, CH), 7.36 (s, 1H, CH), 7.43 (m, 2H, 2 CH), 9.18 (s, 1H, CH), 9.92 (brs, 1H, OH). ^{13}C NMR (75 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 24.8 (CH_2), 25.1 (CH_2), 115.3 (CH), 117.5 (CH), 117.6 (C), 119.0 (CH), 126.9 (C), 129.2 (C), 130.4 (CH), 134.3 (CH), 137.9 (C), 145.8 (C), 146.3 (C), 148.9 (C), 157.9 (C). ES-MS *m*/*z* 323.1 (MH^+). HRMS 323.0961, found 323.0959.

5.1.1.5 3-(2-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8f**

hot NMR

ES-MS *m*/*z* 323.1 (MH^+). HRMS 323.0961, found 323.0956. Anal. Calcd for $C_{17}H_{14}N_4OS$: C 63.33, H 4.38, N 17.38; found: C 62.97, H 4.39, N 17.17.

5.1.1.6 3-(4-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8g**

^{1}H NMR (400 MHz, DMSO) δ: 1.92 (m, 4H, 2 CH_2), 2.91 (m, 2H, CH_2), 3.10 (m, 2H, CH_2), 7.01 (d, *J* = 8.6Hz, 2H, 2 CH), 7.81 (d, *J* = 8.6Hz, 2H, 2 CH), 9.14 (s, 1H, CH), 10.12 (brs, 1H, OH). ^{13}C NMR (100 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 24.8 (CH_2), 25.1 (CH_2), 116.0 (CH), 116.4 (C), 117.8 (C), 129.2 (C), 130.2 (CH), 134.3 (CH), 137.7 (C), 145.9 (C), 146.0 (C), 148.7 (C), 159.4 (C). ES-MS *m*/*z* 323.1 (MH^+). HRMS 323.0961, found 323.0970.

5.1.1.7 3-(4-Hydroxy-3-methoxyphenyl)-5-methyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8h**

^{1}H NMR (400 MHz, DMSO) δ: 1.87 (m, 4H, 2 CH_2), 2.23 (m, 2H, CH_2), 2.86 (m, 2H, CH_2), 3.05 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 6.92 (d, *J* = 8.0Hz, 1H, CH), 7.07 (dd,

J_1 = 8.0Hz, J_2 = 2.0Hz, 1H, CH), 7.24 (d, J = 2.0Hz, 1H, CH), 9.55 (brs, 1H, OH). ES-MS *m/z* 367.1 (MH^+).

5.1.1.8 3-(4-Hydroxy-3-methoxyphenyl)-1,2,4-triazolo[4,3-c]pyrimidine, **8j**
^{1}H NMR (400 MHz, DMSO) δ: 3.88 (s, 3H, OCH_3), 7.01 (t, J = 7.0Hz, 1H, CH), 7.04 (d, J = 8.3Hz, 1H, CH), 7.30 (d, J = 8.3Hz, 1H, CH), 7.42 (m, 2H, 2 CH), 7.83 (d, J = 7.0Hz, 1H, CH), 8.55 (d, J = 7.0Hz, 1H, CH), 9.72 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO) δ: 55.7 (OCH_3), 111.7 (CH), 112.1 (C), 114.3 (CH), 115.4 (CH), 115.9 (CH), 117.2 (C), 121.1 (CH), 124.0 (CH), 127.8 (CH), 146.3 (C), 148.4 (C), 149.5 (C). ES-MS *m/z* 242.0 (MH^+).

5.1.2 *General procedure for oxidative cyclisation of amino imime **11**with aldehydes to give the 1,2,4-triazolo[1,5-c]pyrimidines (**9**,**10a,c,g,k**)*

To a solution of the amino imine **11** [12, 22] (0.25g, 1.13mmol) in methanol (10ml) was added the required aldehyde (1.1 equivalents) and the mixture strirred and heated at reflux overnight. On cooling, the precipitate was isolated by filtration and the solid washed with cold methanol and dried under high vacuum to give the yields shown in Table 5.

5.1.2.1 2-(4-Hydroxy-3-methoxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine **9**
^{1}H NMR (400 MHz, DMSO) δ: 1.91 (m, 4H, 2 CH_2), 2.90 (m, 2H, CH_2), 3.09 (m, 2H, CH_2), 3.90 (s, 3H, OCH_3), 6.95 (d, J = 7.8Hz, 1H, CH), 7.70 9 (s, 1H, CH), 7.71 (d, J = 7.8Hz, 1H, CH), 9.54 (s, 1H, CH), 9.61 (brs, 1H, OH). ^{13}C NMR (100 MHz, DMSO) δ: 21.6 (CH_2), 22.4 (CH_2), 24.8 (CH_2), 24.9 (CH_2), 55.6 (OCH_3), 110.4 (CH), 115.7 (CH), 119.2 (C), 120.8 (CH), 120.9 (C), 128.5 (C), 135.0 (CH), 137.7(C), 147.8 (C), 148.8 (C), 149.2 (C), 152.6 (C), 164.1 (C). ES-MS *m/z* 353.1 (MH^+). HRMS 353.1067, found 353.1069. Anal. Calcd for $C_{18}H_{16}N_4O_2S$: C 61.35, H 4.68, N 15.90; found: C 59.69, H 4.60, N 15.43.

5.1.2.2 2-Phenyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine **10a**
hot NMR
ES-MS *m/z* 307.1 (MH^+). HRMS 307.1012, found 307.1012. Anal. Calcd for $C_{17}H_{14}N_4S$: C 66.64, H 4.61, N 18.29, S 10.47; found: C 66.08, H 4.60, N 18.00, S 10.26.

5.1.2.3 2-(4-Fluorophenyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine **10c**
^{1}H NMR (400 MHz, DMSO) δ: 1.92 (m, 4H, 2 CH_2), 2.92 (m, 2H, CH_2), 3.11 (m, 2H, CH_2), 7.40 (t, J_{HH} = $^3J_{HF}$ = 8.8Hz, 2H, 2 CH), 8.27 (t, J_{HH} = $^3J_{HF}$ = 8.8Hz, 2H, 2 CH), 9.62 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO) δ: 21.6 (CH_2), 22.4 (CH_2), 24.7 (2 CH_2), 116.0 (CH, $^2J_{CF}$ = 21.8Hz), 125.2 (C), 126.4 (C), 128.5 (C), 129.4 (CH, $^3J_{CF}$ = 8.9Hz), 136.9 (CH), 137.2 (C), 138.1 (C), 148.5 (C), 152.7 (C), 162.9 (C). ES-MS *m/z* 325.0 (MH^+). HRMS 235.0918, found 325.0914.

5.1.2.4 2-(4-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine **10g**

^{1}H NMR (400 MHz, DMSO) δ: 1.93 (m, 4H, 2 CH_2), 2.94 (m, 2H, CH_2), 3.15 (m, 2H, CH_2), 6.95 (d, *J* = 8.6Hz, 2H, 2 CH), 8.10 (d, *J* = 8.6Hz, 2H, 2 CH), 9.59 (s, 1H, CH), 10.02 (brs, 1H, OH).

^{13}C NMR (100 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 24.9 (CH_2), 25.0 (CH_2), 115.6 (CH), 119.3 (C), 120.6 (C), 128.6 (C), 128.7 (CH), 136.8 (CH), 137.8 (C), 148.9 (C), 152.7 (C), 159.8 (C), 164.1 (C). ES-MS *m*/*z* 365.1 (MNa^+). HRMS 365.1043, found 365.1056.

5.1.2.5 2-(2-Methylpropyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine **10k**

^{1}H NMR (400 MHz, DMSO) δ: 0.98 (d, *J* = 6.8Hz, 6H, 2 CH_3), 1.89 (m, 4H, 2 CH_2), 2.20 (m, 1H, CH), 2.51 (d, *J* = 6.8Hz, 2H, CH_2), 2.90 (m, 2H, CH_2), 3.04 (m, 2H, CH_2), 9.52 (s, 1H, CH).

hot NMR for 13C?

ES-MS *m*/*z* 287.1 (MH^+). HRMS 287.1325, found 287.1326.

5.2 **Toxin challenge assay**

HeLa cells were pretreated for 30 minutes with growth medium containing 50μM Exo2 or triazole diluted from a 50 mM stock in DMSO or with vehicle DMSO alone, and were then challenged or not for 1h with 50 ng/ml STx in growth medium containing Exo2, compound or DMSO as appropriate. Under these conditions, trial experiments had established that this dose of toxin reduced protein synthesis ability of HeLa cells to 40% of that of non-toxin-treated controls. The remaining ability of the cells to manufacture protein was then assessed by incubating the cells for 30 minutes in PBS containing [^{35}S]-labelled methionine and cysteine, and measuring incorporation of these into acid-precipitable material[9]. The error bars are +/- 1.S.D.

5.3 **Crystallography**

Suitable crystals of **8b**, **9** and **10k** were grown by slow diffusion of methanol into a DMSO solution of the compound in an nmr tube.

The crystals were mounted in oil and the data recorded at 100K using an Oxford Diffraction Gemini four-circle system with Ruby CCD area detector.

The data was solved and refined using the SHELXTL suite of programs [27].

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