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Original article

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

Lucie J. Guetzoyan [a], Robert A. Spooner [b], J. Michael Lord [b], Lynne M. Roberts [b], Guy J. Clarkson [a,*]

[a] Department of Chemistry, University of Warwick, CV4 7AL, UK
[b] Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK

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ABSTRACT

The oxidative cyclisation of a range of benzothieno[2,3-*d*]pyrimidine hydrazones (**7a**–**j**) to the 1,2,4-triazolo[4,3-*c*]pyrimidines (**8a**–**j**) catalysed by lithium iodide or to the 1,2,4-triazolo[1,5-*c*]pyrimidines (**10a**–**j**) with sodium carbonate is presented. A complementary synthesis of the 1,2,4-triazolo[1,5-*c*]pyrimidines 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

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] (Fig. 1).

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 Trans Golgi Network (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 which still contained both the allyl and *n*-propyl ethers but lacked the NH and imine hydrogen of the hydrazone in ^{1}H NMR 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 (Scheme 1).

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

* Corresponding author. Tel.: +44 0 24 765 22255; fax: +44 0 24 76524112.
E-mail address: guy.clarkson@warwick.ac.uk (G.J. Clarkson).

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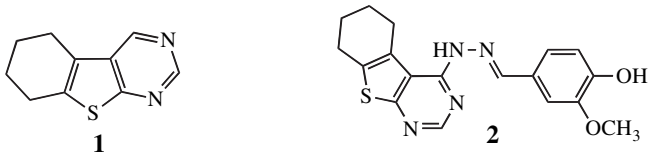


Fig. 1. The 5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidine core (**1**) and Exo2 (**2**).

[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).

We examine other salts as possible additives (Table 1). There is no measurable reaction at room temperature and the oxidative cyclisation in DMSO is slow with low conversion without additive.

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 did not notably enhance the rate, whereas the use of sub-molar quantities had 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 3 shows the range of pyrimidinyl hydrazones **7a–j** that were investigated using the lithium iodide in DMSO reaction conditions. The fluorophenyl hydrazone **7c** and the ester hydrazone **7d** were difficult to cyclise and gave mixtures with longer reaction times leading to decomposition. Hydrazones with a hydroxyphenyl group were the best substrates. The most convenient approach used lithium iodide in DMF as the triazole product 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 (~8.3 ppm/152 ppm in the starting hydrazone) shift to around 9.1 ppm [22] and 135 ppm 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 (Fig. 2).

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 diaminobenzenes 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**).

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 9.54 ppm [12b], but the associated ^{13}C signal still resonates around 135 ppm. 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 X-ray crystallography is shown in Fig. 2 confirming the substitution pattern of the triazole ring (Scheme 3).

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].

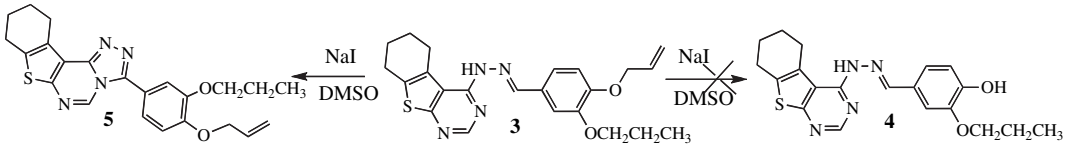
The application of these basic conditions to the range of hydrazones to catalyse the oxidative cyclisation and rearrangement was followed by ^{1}H NMR and is reported in Table 4.

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.

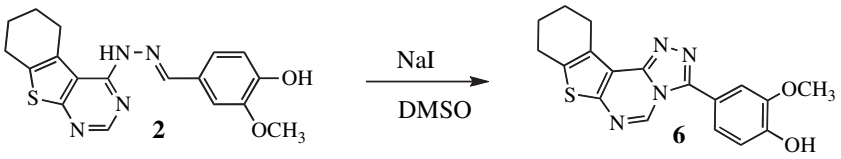
Investigation of the related reaction of amino imine **11** and aldehydes also furnishes the 1,2,4-triazolo[1,5-*c*]pyrimidines (**9**, **10a**, **10c**, **10g**, and **10k**) shown in Table 5.

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

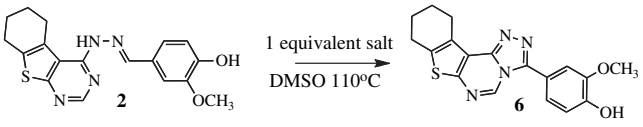


Scheme 1. Oxidative cyclisation during attempted deprotection of allyloxy ether **3**.



Scheme 2. Sodium iodide catalysed oxidative cyclisation of Exo2.

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



Salt	Time	6[a]
none	54 h	25%
NaI	24 h	>99%
NaBr	48 h	31%[b]
NaCl	48 h	24%[b]
Na_2SO_4	48 h	24%
KI	48 h	>99%
LiI	18 h	>99%
NMe_4I	48 h	>99%

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

hydrazone. Simple filtration gives good yields for both electron rich and electron deficient benzaldehydes and aliphatic aldehydes.

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 of **2** to **6** 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 hydrazone **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.

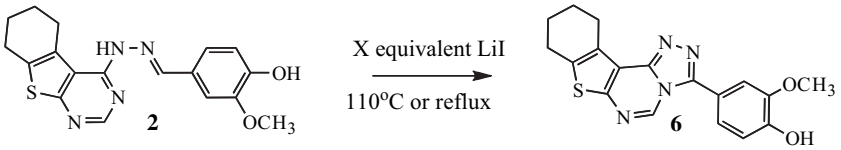
We finally looked at the reaction of **2** with an equivalent of iodine in DMSO which does lead to a rapid oxidative cyclisation but to the isomeric 1,2,4-triazolo[1,5-*c*]pyrimidine with concomitant iodinisation to give isomer **13** based on the coupling constant between the two remaining hydrogens on the electron rich phenolic ring.

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 (Scheme 5) 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 stabilize a radical intermediate in the 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

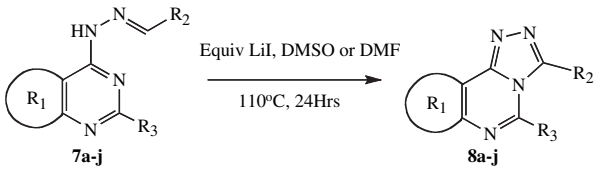
Table 2
Influence of the solvent on LiI catalysed oxidative cyclisation of Exo2.



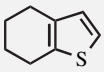
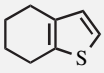
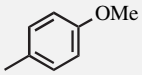
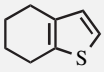
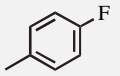
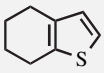
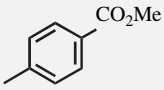
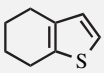
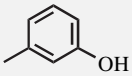
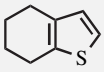
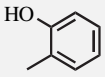
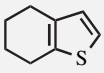
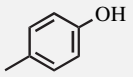
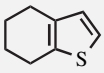
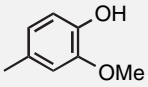
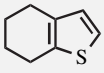
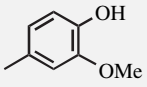
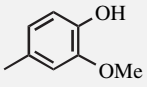
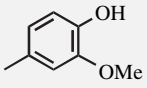
Solvent	LiI (equiv.)	Time	2[a]	6[a]
DMF	1	28 h	–	78%[b]
DMF	0	48 h	–	69%[b]
DMSO	1	24 h	–	>99%
DMSO	0	54 h	75%	25%
MeOH	1	48 h	>99%	–
EtOAc	1	48 h	83%	17%
acetone	1	48 h	>99%	–
CH_3CN	1	48 h	5%[c]	95%[c]
CH_3CN	0	48 h	>99%[c]	+
NO_2CH_3	1	48 h	>99%	–

[a] Ratio determined by NMR.
[b] Isolated yield.
[c] Precipitate collected and NMR recorded in DMSO(>80% recovery).

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**	70%[c]	–
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.

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 **10g** (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].

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 (Fig. 3 Graph A, light grey bars). The procedure was replicated but this time, the cells were challenged for 1 h 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 (Fig. 3 Graph A, dark grey bars). Most of the triazoles were substantially less toxic than Exo2 in this regard, although **10g** 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, dark grey versus light grey bars). The protective effect (Fig. 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 induce the oxidative cyclisation of pyrimidine hydrazones to 1,2,4-triazolo[4,3-*c*]pyrimidines by heating in DMSO or DMF. Lithium iodide is an effective additive to increase the reaction rate and yield 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.

4.1. Experimental

All reagents and solvents were purchased from Lancaster and Aldrich and used without further purification. The hydrazone

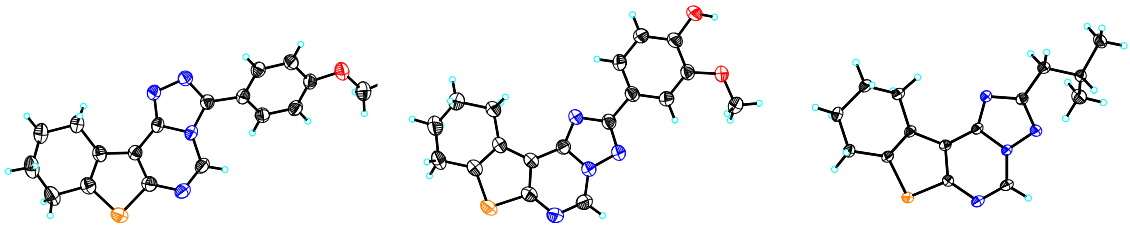


Fig. 2. Solid state structure of **8b**, **9** and **10k** with thermal ellipsoids drawn at 50% probability.

starting materials were synthesised as detailed elsewhere [9]. NMR spectra were recorded on a DPX-400 spectrometer at room temperature (298 K). The spectra were recorded in parts per million (ppm) and referenced using residual protio solvents relative to trimethylsilane standard ($\delta_H = 0$ ppm). 2D COSY, HMQC, HMBC and NOESYspectra 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. CHN elemental analyses were carried out by Warwick Analytical Services. Thin Layer Chromatography used in monitoring reaction progress were performed using silica layer (0.25 mm) coated alumina plates. Weights were recorded on a balance to 4 decimal places.

*4.1.1. General procedure for lithium iodide catalysed oxidative cyclisation of pyrimidyl hydrazones to give the 1,2,4-triazolo-[4,3-c]pyrimidines (**6**,**8a**–**j**)*

The hydrazone (5mmole) was dissolved in reagent grade DMF (15 ml) and one equivalent of lithium iodide was added (1 equiv., 0.7 g). The mixture was stirred and heated at 110 °C under an air atmosphere. After the specified time (24 or 48 h), 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.

*4.1.1.1. 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, $J = 8.0$ Hz, 1H, CH), 7.39 (dd, $J_1 = 8.0$ Hz, $J_2 = 2.0$ Hz, 1H, CH), 7.50 (d, $J = 2.0$ Hz, 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 4.58, N 15.90; found: C 61.35, H 4.51, N 15.57.

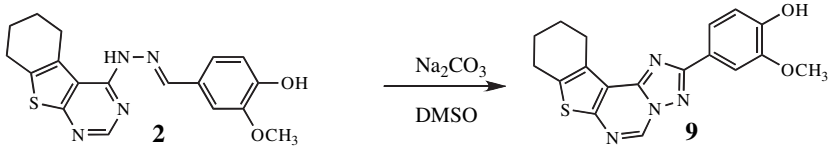
*4.1.1.2. 3-Phenyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8a**.* ^{1}H NMR (400 MHz, DMSO) δ: 1.94 (m, 4H, 2 CH_2), 2.93 (m, 2H, CH_2), 3.13 (m, 2H, CH_2), 7.66 (m, 3H, 3 CH), 8.01 (m, 2H, 2 CH), 9.22 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO) δ: 21.7 (CH_2), 22.5 (CH_2), 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. m.p. 185–186 °C. Anal. Calcd for $C_{17}H_{14}N_4S$: C 66.64, H 4.61, N 18.29; found: C 66.31, H 4.57, N 17.96.

*4.1.1.3. 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, $J = 8.8$ Hz, 1H, CH), 7.93 (d, $J = 8.8$ Hz, 1H, 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. m.p. 213–215 °C. Anal. Calcd for $C_{18}H_{16}N_4OS$: C 64.26, H 4.49, N 16.65, S 9.53; found: C 63.89, H 4.78, N 16.51, S 9.34.

*4.1.1.4. 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, $J = 7.6$ Hz, 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). m.p. = 252–255 °C. ES-MS m/z 323.1 (MH^+). HRMS 323.0961, found 323.0959.

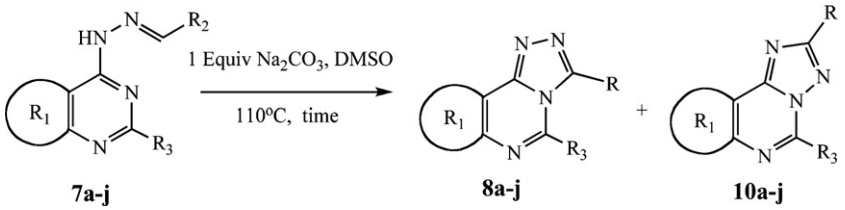
*4.1.1.5. 3-(2-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8f**.* ^{1}H NMR (400 MHz, DMSO spectra recorded at 353 K) δ: 1.91 (m, 4H, 2 CH_2), 2.90 (m, 2H, CH_2), 3.10 (m, 2H, CH_2), 7.07 (t, $J = 8.1$ Hz, 1H, CH), 7.13 (d, $J = 8.1$ Hz, 1H, CH), 7.50 (t, $J = 8.1$ Hz, 1H, CH), 7.63 (d, $J = 8.1$ Hz, 1H, CH), 8.84 (s, 1H, CH), 10.57 (brs, 1H, OH). ES-MS m/z 323.1 (MH^+). HRMS 323.0961, found 323.0956. m.p. = decomposes above 260 °C. 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.

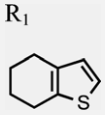
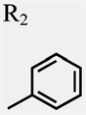
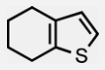
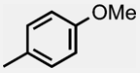
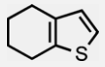
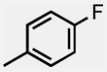
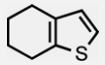
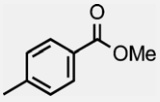
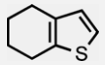
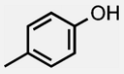
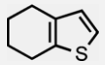
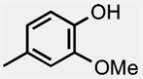
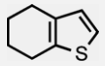
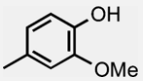
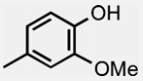
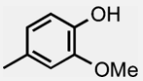
*4.1.1.6. 3-(4-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine **8g**.* ^{1}H NMR (400 MHz, DMSO) δ: 1.92 (m, 4H, 2 CH_2), 2.91 (m, 2H, CH_2), 3.10 (m, 2H, CH_2), 7.01 (d, $J = 8.6$ Hz, 2H, 2 CH), 7.81 (d, $J = 8.6$ Hz, 2H, 2 CH), 9.14 (s, 1H, CH), 10.12 (brs, 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



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

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.**



	R_1	R_2	R_3	X	Time	Product	Ratio %[a]
7a			H	N	2d[b]	**7a** **8a** **10a**	50% 50% –
7b			H	N	2d[b]	**7b** **8b** **10b**	20% – 80%
7c			H	N	5d[b]	**7c** **8c** **10c**	30% 5% 65%
7d			H	N	24 h[b]	**7d** **8d** **10d**	– – >90%
7g			H	N	4d	**7g** **8g** **10g**	– – >99%
2			H	N	2d	**2** **6** **9**	– – >99%
7h			CH_3	N	3d	**7h** **8h** **10h**	– – >99%
7i			H	N	4d[b]	**7i** **8i** **10i**	45% 10% 45%
7j	H–, H–		H	C	4d[b]	**7j** **8j** **10j**	45% 10% 45%

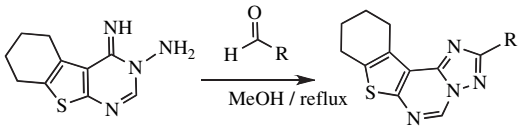
[a] Ratio determined by NMR.
[b] Further heating leads to decomposition.

(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. m.p. > 300 °C. Anal. Calcd for $C_{17}H_{14}N_4OS$: C 63.33, H 4.38, N 17.38; found: C 62.88, H 4.43, N 16.98.

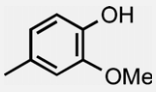
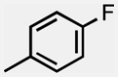
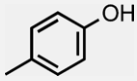
4.1.1.7. 3-(4-Hydroxy-3-methoxyphenyl)-5-methyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[4,3-c]pyrimidine ***8h****.* ^{1}H NMR (400 MHz, DMSO) δ: 1.87 (m, 4H, 2 CH_2), 2.23 (m, 2H, CH_2), 2.86 (m, 2H, CH_2), 3.05 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 6.92 (d, J = 8.0 Hz, 1H, CH), 7.07 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H, CH), 7.24 (d, J = 2.0 Hz, 1H, CH), 9.55 (brs, 1H, OH). ES-MS *m*/*z* 367.1 (MH^+).

4.1.1.8. 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 = 7.0 Hz, 1H, CH), 7.04 (d, J = 8.3 Hz, 1H, CH), 7.30 (d, J = 8.3 Hz, 1H, CH), 7.42 (m, 2H, 2 CH), 7.83 (d, J = 7.0 Hz, 1H, CH), 8.55 (d, J = 7.0 Hz, 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),

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



11 **9, 10a, 10c, 10g, 10k**

Product	R	Yield
9		65%
10a		66%
10c		51%
10g		34%
10k		70%

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^+).

4.1.2. 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] (0.25 g, 1.13 mmol) in methanol (10 ml) was added the required aldehyde (1.1 equiv) and the mixture stirred 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.

4.1.2.1. 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 = 7.8 Hz, 1H, CH), 7.70 9 (s, 1H, CH), 7.71 (d, J = 7.8 Hz, 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. m.p. = 254–256 °C. Anal. Calcd for $C_{18}H_{16}N_4O_2S$: C 61.35, H 4.58, N 15.90; found: C 59.69, H 4.60, N 15.43.

4.1.2.2. 2-Phenyl-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine ***10a***. ^{1}H NMR (300 MHz, DMSO) δ: 1.92 (m, 4H, 2 CH_2), 2.94 (m, 2H, CH_2), 3.17 (m, 2H, CH_2), 7.57 (m, 3H, 3 CH), 8.27 (m, 3H, 3 CH), 9.67 (s, 1H, CH). 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 66.48, H 4.60, N 18.00, S 10.26.

4.1.2.3. 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} = 3, J_{HF} = 8.8 Hz, 2H, 2 CH), 8.27 (t, J_{HH} = 3, J_{HF} = 8.8 Hz, 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, $^2J_{CF}$ = 21.8 Hz), 125.2 (C), 126.4 (C), 128.5 (C), 129.4 (CH, $^3J_{CF}$ = 8.9 Hz), 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.

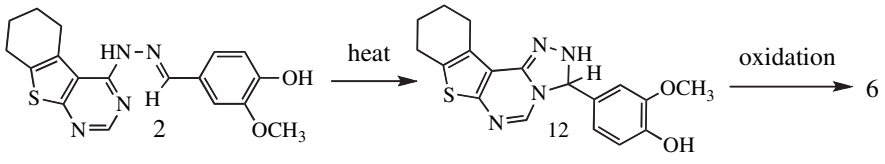
4.1.2.4. 2-(4-Hydroxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine ***10g***. ^{1}H NMR (400 MHz, DMSO) δ: 1.93 (m, 4H, 2 CH_2), 2.94 (m, 2H, CH_2), 3.15 (m, 2H, CH_2), 6.95 (d, J = 8.6 Hz, 2H, 2 CH), 8.10 (d, J = 8.6 Hz, 2H, 2 CH), 9.59 (s, 1H, CH), 10.02 (brs, 1H, 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. m.p. decomposes at 250 °C. Anal. Calcd for $C_{17}H_{14}N_4OS$: C 63.33, H 4.38, N 17.38; found: C 62.93, H 4.45, N 16.98.

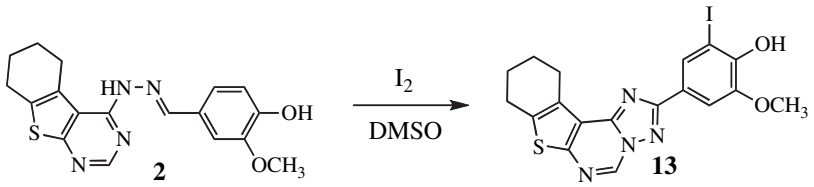
4.1.2.5. 2-(2-Methylpropyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine ***10k***. ^{1}H NMR (400 MHz, DMSO spectra recorded at 353 K) δ: 0.98 (d, J = 6.8 Hz, 6H, 2 CH_3), 1.89 (m, 4H, 2 CH_2), 2.20 (m, 1H, CH), 2.51 (d, J = 6.8 Hz, 2H, CH_2), 2.90 (m, 2H, CH_2), 3.04 (m, 2H, CH_2), 9.52 (s, 1H, CH).

^{13}C NMR (100 MHz, DMSO, 353 K) δ: 22.2 (CH_2), 22.8 (2 CH_3), 23.0 (CH_2), 25.3 (CH_2), 25.4 (CH_2), 27.9 (CH), 37.7 (CH_2), 119.9 (C), 129.0 (C), 136.9 (CH), 138.4 (C), 148.9 (C), 153.0 (C), 167.6 (C). ES-MS *m*/*z* 287.1 (MH^+). HRMS 287.1325, found 287.1326. m.p. = 121–122 °C. Anal. Calcd for $C_{15}H_{18}N_4S$: C 62.91, H 6.33, N 19.56, S 11.20; found: C 62.50, H 6.26, N 19.42, S 11.18.

4.1.2.6. 2-(4-Hydroxy3-iodo-5-methoxyphenyl)-8,9,10,11-tetrahydro[1]benzothieno[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine ***13***. ^{1}H NMR (400 MHz, DMSO) δ: 1.94 (m, 4H, 2 CH_2), 2.91 (m, 2H, CH_2), 3.08 (m, 2H, CH_2), 3.98 (s, 3H, OCH_3), 5.10 (brs, OH in water peak), 7.65 (d, J = 1.8 Hz, 1H, CH), 8.11 (d, J = 1.8 Hz, 1H, CH), 9.52 (s, 1H, CH). ^{13}C NMR (100 MHz, DMSO) δ: 21.6 (CH_2), 22.4 (CH_2), 24.8 (2 CH_2), 84.5 (C), 109.6 (CH), 119.2 (C), 122.5 (C), 128.5 (C), 129.2 (CH), 137.9 (C), 147.0 (C), 148.6 (C), 152.7 (C), 162.5 (C). ES-MS *m*/*z* 479.0 (MH^+).



Scheme 4. Possible diamino acetal intermediate in the oxidative cyclisation of Exo2.



Scheme 5. Iodine catalysed oxidation and rearrangement of Exo2.

4.2. Toxin challenge assay

HeLa cells were pretreated for 30 min with growth medium containing 50 μM Exo2 or triazole diluted from a 50 mM stock in DMSO or with vehicle DMSO alone, and were then challenged or not for 1 h 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 min 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.

4.3. 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 with glue or oil and the data recorded 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].

The supplementary crystallographic data for this paper can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif quoting the appropriate CCDC number.

Crystal data for (**8b**) $C_{18}H_{16}N_4OS$, $M = 336.42$, colourless rod, 0.50 × 0.08 × 0.06 mm, Monoclinic, P2(1) (No 4), $\beta = 98.953(2)^\circ$, $a = 5.3889(1)$, $b = 18.1190(3)$, $c = 8.1673(2)$ Å, $T = 296(2)$K, μ(Mo-K_α) = 0.077 mm^{-1}, $U = 787.75(3)$ Å^3, Z 2, $D_{cal} = 1.418$ g cm^{-3}, Flack 0.01(5) for 1688 Friedel pairs, 21436 reflections measured, 3945 unique [$R_{int} = 0.0362$], R [$I > 2\sigma(I)$] = 0.0467, wR [$I > 2\sigma(I)$] = 0.0808, $GooF = 1.016$, CCDC 741336.

Crystal data for (**9**) $C_{18}H_{16}N_4O_2S$, $M = 352.42$, yellow block, 0.25 × 0.20 × 0.10 mm, Monoclinic, P2(1)/c (No 14), $\beta = 90.3577(17)^\circ$, $a = 5.85370(10)$, $b = 12.5466(2)$, $c = 21.7465(4)$ Å, $T = 296(2)$K, μ(Mo-K_α) = 0.077 mm^{-1}, $U = 1597.12(5)$ Å^3, Z 4, $D_{cal} = 1.466$ g cm^{-3}, 18182 reflections measured, 2928 unique [$R_{int} = 0.0435$], R [$I > 2\sigma(I)$] = 0.0659, wR [$I > 2\sigma(I)$] = 0.1182, $GooF = 1.073$, CCDC 741337.

Crystal data for (**10k**) $C_{15}H_{18}N_4S$, $M = 286.39$, yellow block, 0.40 × 0.10 × 0.10 mm, Monoclinic, P2(1)/n (No 14), $\beta = 102.517(2)^\circ$, $a = 13.2799(3)$, $b = 5.24816(9)$, $c = 20.1671(4)$ Å, $T = 100(2)$K, μ(Mo-K_α) = 0.077 mm^{-1}, $U = 1372.14(5)$ Å^3, Z 4, $D_{cal} = 1.386$ g cm^{-3}, 16734 reflections measured, 4653 unique [$R_{int} = 0.0353$], R [$I > 2\sigma(I)$] = 0.0390, wR [$I > 2\sigma(I)$] = 0.0947, $GooF = 0.972$, CCDC 741338.

All non hydrogen atoms were refine anisotropically. Hydrogen atoms were placed at calculated positions except the OH in **9** which was located in a difference map. It's position was allowed to refine freely but given thermal parameters equal to 1.5 times the oxygen to which it was attached.

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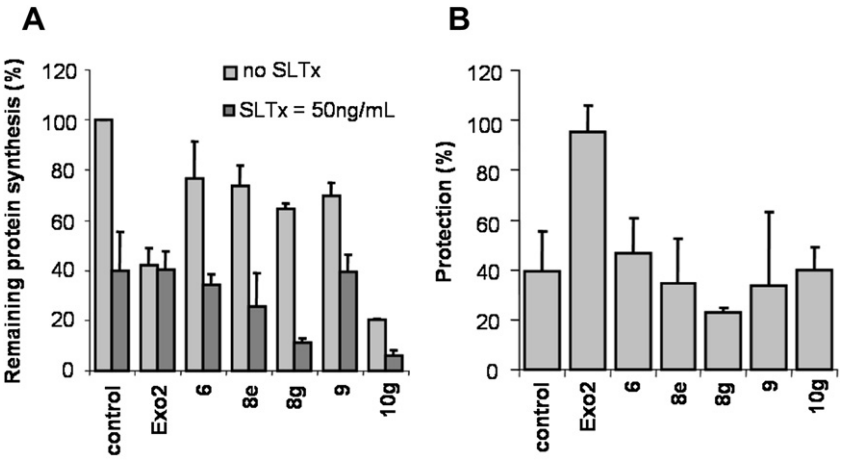

Table A – protein synthesis on treatment with compound (light grey bars) and on treatment with compound and STx (dark grey bars) normalised to carrier DMSO
Table B – protective effect as described by the ratio of protein synthesis in the presence of the test compound with and without STx challenge

Fig. 3. Biological activity of the triazolopyrimidines in Shiga Toxin Assay.

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