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Synthesis of novel 5-amino-thiazolo[4,5-*d*]pyrimidines as *E. coli* and *S. aureus* SecA inhibitors

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ABSTRACT

An efficient synthesis of a library of 5-amino-thiazolo[4,5-*d*]pyrimidines is reported. Regioselective displacements of chlorines, as well as regioselective diazotation reactions are described, which allow the introduction of structural diversity on the scaffold by consecutive reactions. Screening of this focused library led to the discovery of SecA inhibitors from *Escherichia coli* and *Staphylococcus aureus*.

1. Introduction

After the discovery of penicillin by Alexander Fleming, antibiotics were regarded as wonder drugs for curing virtually all infections. However, the careless use and overconsumption of antibiotics in both human and veterinary medicine has led to the emergence of antibiotic-resistant bacterial strains. Of major concern is the development of antibiotic resistance in *Staphylococcus aureus*, primarily because *S. aureus* is frequently associated with hospital and community-acquired infections. Infections with multi-drug resistant *S. aureus* have become responsible for huge healthcare costs and are projected to be responsible for more deaths this year in the United States than HIV/AIDS.[1] Despite this increasing problem of antibiotic resistance, the number of different antibiotics available is dwindling and there are only a handful of new antibiotics in the drug development pipeline.[2] Therefore, there is an urgent need for new antibacterial drugs preferably with new modes of action to potentially avoid cross-resistance.[3] In the framework of the search for new therapies, new antibiotic targets are currently being proposed and evaluated, including components of the bacterial protein secretion pathways.[4,5]

The major route for the transport of bacterial proteins across or into the bacterial cytoplasmic membrane is provided by the Sec pathway.[6] A key component of the Sec transport system is the peripheral membrane ATPase SecA, which couples the hydrolysis of ATP to the stepwise translocation of preproteins across the bacterial cytoplasmic membrane.[7,8] Because SecA is a conserved and essential protein in all bacteria but is absent in humans, it is considered as a promising antibacterial drug target.[4,9]

However, only a very limited number of SecA inhibitors have been described so far. Sodium azide displays SecA inhibitory activity with an IC_{50} of 1 mM.[10] The natural compound CJ-21058, isolated from the fermentation broth of an unidentified fungus, showed antibacterial activity against Gram-positive multi-drug resistant bacteria by inhibiting the SecA translocation ATPase activity.[11] Researchers from Merck used a strain of *S. aureus* that generates an antisense RNA against SecA to screen for novel antibacterials. By this strategy, they were able to isolate a new *cis*-decalin metabolite, which they named pannomycin.[12] Scientists from Wyeth developed an assay that makes use of a SecA-LacZ fusion reporter construct in *Escherichia coli*, which is induced when secretion is perturbed. Several compounds have been discovered that turned out to be secretion inhibitors. However, they were also found to have deleterious effects on membranes.[13] In 2008, researchers reported a structure-based virtual screening approach using the published X-ray structure of *E. coli* SecA[14] for the discovery of small-molecule inhibitors of the intrinsic ATPase activity of SecA.[15] The most potent compounds had IC_{50} values of about 100 μM. Later on, the same research group reported a hit-to-lead optimization process in which they could improve the biological activity yielding analogues with IC_{50} of 20–60 μM.[16]

There is a very limited number of heterocyclic, drug-like scaffolds in literature known to be as SecA inhibitors. However, from

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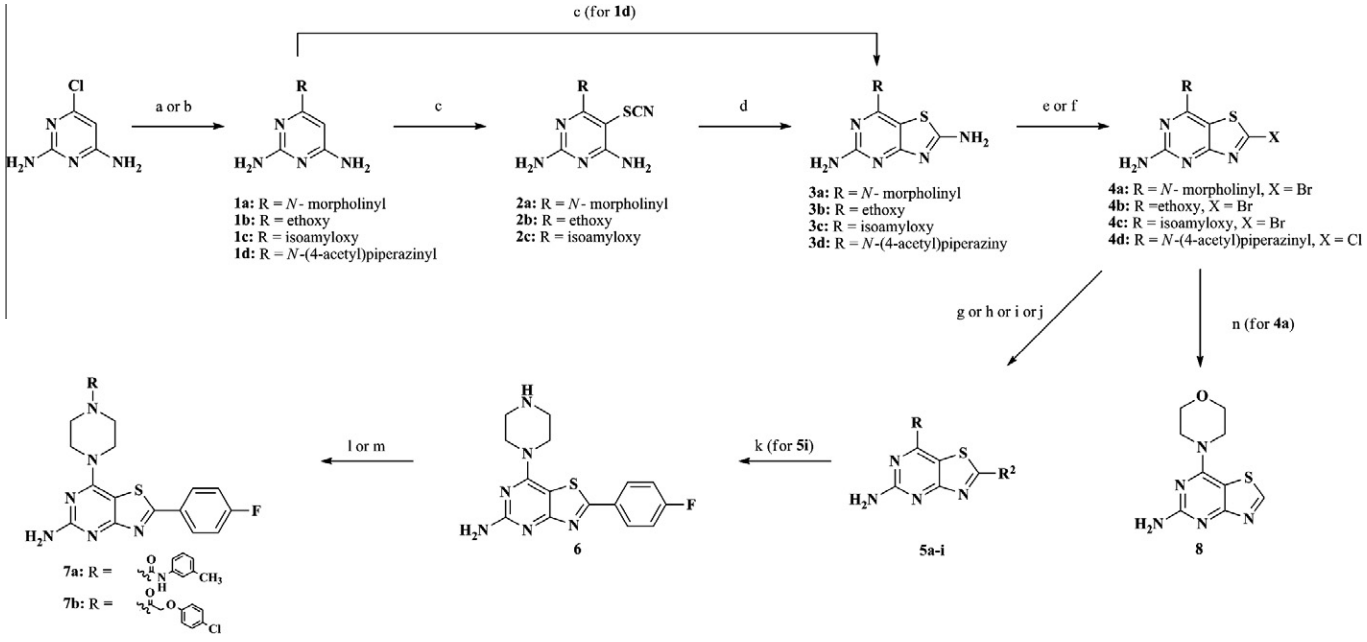
a drug discovery standpoint of view, it is important to have access to different heterocyclic structures. The availability of different chemotypes offers a choice in terms of chemical accessibility and prospects for lead optimization. As each heterocyclic scaffold has its own characteristics regarding pharmacology, pharmacokinetics and toxicology, it is important to have access to a wide variety of different chemistries. In addition, backup compounds lower the chance of attrition in later phases of drug development. A very common strategy in order to find new chemistry starting point is a high-throughput screening (HTS) campaign of commercially available compound libraries against the target of interest. Although the number of available compounds for HTS has dramatically increased over the last few years, large-scale random combinatorial libraries have contributed proportionally less to the identification of novel hits or leads. Especially for antibacterial HTS campaigns, the success rate is four to fivefold lower than for targets from other therapeutic areas.[17] The results of these HTS campaign were disappointing. An often cited reason for this lack of productivity is the lack of chemical diversity in the available compound libraries. The only way to address this issue involves the synthesis of novel (i.e., non-commercially available) compound libraries, based on unexplored chemistry. The structures of most marketed antibiotics do not obey general rules formulated for drug-likeness (such as the Lipinski rules). This is due to the complex structure of the cell wall of bacteria, the particular structure of the bacterial targets and the mode of action which is needed to generate a bacteriostatic or bactericidal effect. SecA is a peripheral membrane ATPase: it adheres only temporarily with the bacterial membrane (i.e., when SecA is actively involved in preprotein translocation). Up to now, it seems to be refractory to inhibitor design. Therefore, we have conducted the synthesis (and the use for SecA screening) of novel scaffolds that have been hardly explored in antibacterial drug discovery, primarily based on the chemistry of thiazolo[4,5-*d*]pyrimidines. Compounds based on this scaffold are not very well represented in commercial compound libraries and, although this scaffold is not frequently used in drug discovery programs, some interesting biological activity has been associated with this scaffold. Thiazolo[4,5-*d*]pyrimidine derivatives, prepared as guanine mimics, showed potent in vitro activity against human cytomegalovirus (HCMV).[18] Thiazolo[4,5-*d*]pyrimidine-5,7-dione analogues have been reported as having anti-inflammatory activities, due to TNF inhibition.[19] 2-Oxo-3-aryl-thiazolo[4,5-*d*]pyrimidine analogues have been synthesized as antagonists of the corticotrophin releasing hormone (CRH) R_1 receptor.[20] 2-Thio-3-aryl-thiazolo[4,5-*d*]pyrimidine derivatives have been described as having anticancer,[21] anti-inflammatory and antimicrobial activity.[22] 2-Aminothiazolo[4,5-*d*]pyrimidines, that act as $CXCR_2$ receptor antagonists are also known.[23] Recently, 2,7-substituted-thiazolo[4,5-*d*]pyrimidines have been described as ATP-competitive inhibitors of several protein kinases (EGFR, cSrc, HER-2, Lyn and c-Abl).[24]

These initial data demonstrate that thiazolo[4,5-*d*]pyrimidines display a plethora of biological activities and this scaffold can therefore be considered as a privileged structure.[25] Priviliged structures represent a class of molecules capable of binding to multiple target proteins (receptors or enzymes) with high affinity. The exploitation of these molecules allows the medicinal chemist to rapidly discover biologically active compounds across a broad range of therapeutic areas. In order to fully explore the biological activity of this scaffold, it is necessary to have access to synthetic schemes that can be used for the construction of compound libraries. To our knowledge, there are no systematic studies done on how to elaborate in a systematic way the chemistry of this compound class. Therefore, the main goal of this research is to develop synthetic schemes that can be easily adapted for use in parallel chemistry, and hence are suitable to establish structure–activity relationship (SAR) studies. We introduced a broad structural variety into the thiazolo[4,5-*d*]pyrimidine scaffold in which different substitution sites can be varied in one synthetic cycle. In this paper, we wish to report our findings towards the elaboration of this type of chemistry and its application for the discovery of novel SecA inhibitors.



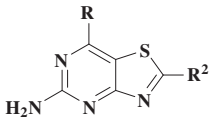
Scheme 1. Synthesis of 5-amino-thiazolo[4,5-*d*]pyrimidines. Reagents and conditions: (a) alcohol, NaH, reflux; (b) amine, water, reflux; (c) KSCN, pyridine, Br_2, DMF, 65–5 °C; (d) DMF/water (4:1 v/v), 140 °C; (e) isoamyl nitrite, TMSBr, CH_3CN, rt; (f) $NaNO_2$, concd H_2SO_4, 80 °C then concd HCl, CuCl, 40 °C; (g) boronic acid, K_2CO_3, $Pd(PPh_3)_4$, dioxane/water (3:1 v/v), 100 °C; (h) tributylphenylstannane, $Pd(PPh_3)_4$, reflux; (i) phenylacetylene, $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, dioxane, 80 °C; (j) amine, NEt_3, dioxane, rt; (k) 5% HCl, 100 °C; (l) *m*-tolyl isocyanate, DMF, rt; (m) *p*-chlorophenoxyacetyl chloride, pyridine, DMF, rt.; (n) H_2, 10% Pd/C, THF/MeOH (2:1 v/v), rt.

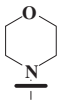
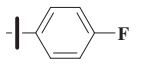
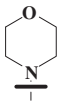
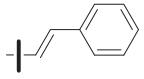
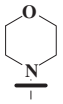
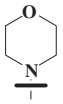
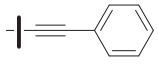
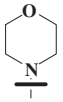
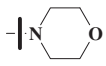
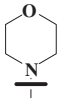
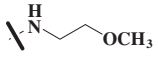
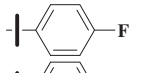
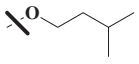
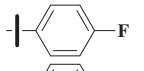
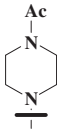
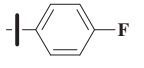
2. Chemistry

In order to have access to synthetic strategies that can be used in high-throughput medicinal chemistry labs, it is desirable to use a multi-halogenated thiazolo[4,5-*d*]pyrimidine as a key intermediate from which, in several consecutive steps, structural variety can be introduced. They can react with a wide range of suitable nucleophiles, but can also be used for a variety of palladium-mediated cross-coupling reactions such as Hartwig–Buchwald, Heck, Negishi, Sonogashira, Stille and Suzuki couplings. Therefore, halogenated heterocycles are valuable scaffolds for the design of heterocyclic libraries and in addition, they are useful tools to investigate chemical reactivity.

The synthesis of thiazolo[4,5-*d*]pyrimidine analogues starts from commercially available 2,6-diamino-6-chloro-pyrimidine. The 2,6-diamino-4-substituted pyrimidine analogues **1a–d** are easily obtained from commercially available 2,6-diamino-6-chloro-pyrimidine by known procedures (Scheme 1). The desired alkoxy groups were introduced by refluxing 2,6-diamino-4-chloropyrimidine in the appropriate alcohol with sodium hydride,[26] whereas for the introduction of morpholine and *N*-acetylpiperazine, water was chosen as solvent.[27] Thiocyanation of the 2,6-diamino-4-substituted pyrimidines **1a–d** by treatment with potassium thiocyanate, bromine and pyridine in *N*,*N*-dimethylformamide (DMF), followed by refluxing in DMF/water, afforded the 2,5-diamino-7-substituted-thiazolo[4,5-*d*]pyrimidine analogues **3a–c**.[28] For the *N*-acetyl-piperazine analogue **1d**, a direct formation of the thiazole ring was observed (yielding compound **3d**), without the need of heating at 140 °C. In order to introduce structural variety at position 2, the 2-amino group was converted to bromine via diazotation of **3a–c** by treatment with isoamyl nitrite and bromotrimethylsilane in acetonitrile.[23a] For the 7-*N*-acetylpiperazine derivative **4d**, we opted for the introduction of a chlorine using diazotation, followed by chlorination with CuCl (Sandmeyer reaction). The 2-halo (bromine or chlorine) substituted heterocycle is a versatile starting material for a wide variety of palladium catalyzed cross-coupling reactions to form new C–C bonds. Standard reaction conditions for a Suzuki coupling (method g),[29] a Stille coupling (method h),[30] a Sonogashira coupling (method i)[31] and a nucleophilic aromatic substitution reaction (method j)[32] of compounds **4a–d** afforded the desired compounds **5a–i** in moderate to good yields (Table 1). The acetyl group of 7-((*N*-acetyl)piperazin-1-yl)thiazolo[4,5-*d*]pyrimidine **5i** was cleaved off under acidic conditions, and the piperazine moiety **6** was coupled with *m*-tolyl isocyanate (yielding urea **7a**) or with *p*-chlorophenoxyacetyl chloride (affording amide **7b**).

Table 1
Overview of the thiazolo[4,5-*d*]pyrimidines



Reactant	Method	Product	R	R^2	Yield (%)
4a	g	**5a**			88
4a	g	**5b**			32
4a	h	**5c**			50
4a	i	**5d**			89
4a	j	**5e**			86
4a	j	**5f**			93
4b	g	**5g**			86
4c	g	**5h**			76
4d	g	**5i**			73

The presence of two amino groups for the diazotation procedure presents a potential liability, regarding regioselectivity. Using compound **4a** as representative example, the selective diazotation of the 2-amino group was proven. The bromine was cleaved off by catalytic hydrogenation, yielding 5-amino-7-*N*-morpholinothiazolo[4,5-*d*]pyrimidine **8**. The assignment of C(7) at 159 ppm in the ^{13}C NMR spectrum was staightforward by the observation of a HMBC correlation (^{3}J) between the protons of the morpholino moiety and C(7) (Fig. 1). A clear HMBC cross-peak was seen between C(7a) (96 ppm) and the proton at 9.5 ppm (^{3}J), which is only possible if this signal is due to a proton at position 2 of the scaffold **8**. This confirms the presence of the hydrogen at position 2 of the scaffold and hence, proves that the diazotation preferentially takes place at the 2-amino group. This observation is in clear agreement with previous results in which a selective diazotation of the amino group at position 2 of 2,5-diaminothiazolo[4,5-*d*]pyrimidin-7-(6*H*)one has been described and was proven by chemical transformation.[33]

To introduce a variety of substituents at positions 2 and 7 of the 5-amino-thiazolo[4,5-*d*]pyrimidine scaffold, a 2,7-dihalo-thiazolo[4,5-*d*]pyrimidine was considered as a ideal key intermediate. If the two halogen atoms show different reactivity, this characteristic can then be exploited for sequential introduction of different substituents. The 5-amino-2,7-dichlorothiazolo[4,5-*d*]pyrimidine was synthesized from the known 5-amino-2-chlorothiazolo[4,5-*d*]pyrimidin-7(6*H*)one **9** (Scheme 2).[33] The lactam functionality of compound **9** was chlorinated by refluxing in $POCl_3$ with *N*,*N*-dimethylaniline,[34] yielding a 5-amino-2,7-dichlorothiazolo[4,5-*d*]pyrimidine **10**. It was expected that the chlorine at position 7 would be more reactive. It is indeed known that for similar heterocycles, such as pyrido[3,2-*d*]pyrimidines[35] and pyrido[2,3-*d*]pyrimidines,[36] the chlorine at position 4 of the scaffold is extremely labile and can be easily displaced by nucleophiles. Moreover, when a closely related 6,8-dichloropurine was subjected to a Stille coupling, selective coupling at the 6 position was achieved.[37] However, reaction of **10** with 1 equiv of a nucleophile allowed selective substitution of chlorine at position 2. Two representative examples of nucleophiles were selected: a primary amine (methoxyethylamine) and a secondary amine (*N*-Boc-piperazine). The subsequent Suzuki coupling of **11a** and **11b** with 4-fluorophenylboronic acid afforded compounds **12a** and **12b**. The Boc group of **12b** was cleaved off under acidic conditions, and the piperazine moiety **13** was further derivatised with *m*-tolyl isocyanate or *p*-chlorophenoxyacetyl chloride affording compounds **14a** and **14b**, respectively.

In order to prove the regiochemistry of the nucleophilic aromatic substitution reaction, the remaining chlorine of compound **11b** was cleaved off catalytically using hydrogen and palladium as catalyst. The obtained compound turned out to be very insoluble in common organic solvents, and therefore, the amino group was acetylated yielding compound **15**, which is easier to handle. The structure of compound **15** was solved by Heteronuclear Multiple Bond Correlation (HMBC) spectroscopy (Fig. 1). The ^{13}C signal at $\delta = 170$ ppm, arising from C(5), was used as a starting point in the structural elucidation. A clear HMBC cross-peak was seen between C(5) and the proton at 8.7 ppm, which is only possible if this signal is due to a proton at position 7 of the scaffold. A direct coupling (^{1}J) of H(7) with C(7) is observed, making the assignment of C(7) at $\delta = 149$ ppm possible. Also, clear HMBC correlations are found between H(7) and C(7a) (^{2}J) and C(3a) (^{3}J). These findings

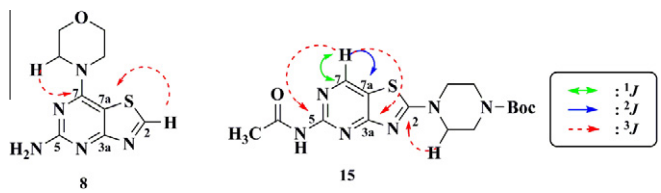
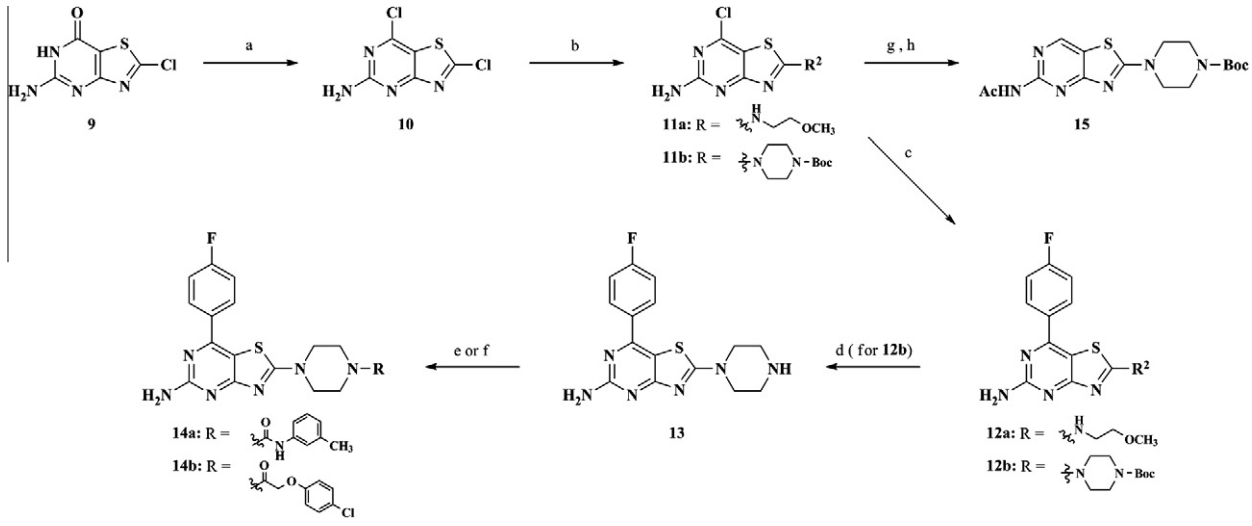
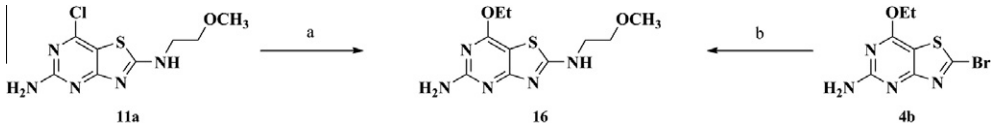


Figure 1. Structure elucidation of compounds **8** and **15**.



Scheme 2. Synthesis of 5-amino-7-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidines. Reagents and conditions: (a) $POCl_3$, *N*,*N*-dimethylaniline, reflux; (b) amine, NEt_3, dioxane, 100 °C; (c) 4-fluorophenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, dioxane/water (3:1 v/v), 100 °C; (d) TFA, CH_2Cl_2, rt; (e) *m*-tolylisocyanate, DMF, rt; (f) *p*-chlorophenoxyacetyl chloride, pyridine, DMF, rt; (g) H_2, 10% Pd/C, THF/MeOH (2:1 v/v), rt; (h) Ac_2O, reflux.



Scheme 3. Determination of regioselectivity via chemical synthesis. Reagents and conditions: (a) NaH, EtOH, reflux; (b) 2-methoxyethylamine, dioxane, 60 °C.

prove that the *N*-Boc-piperazinyl group is present at position 2, whereas C(7) bears a hydrogen atom.

Having established a reliable synthetic scheme for the preparation of 5-amino-2,7-disubstituted thiazolo[4,5-*d*]pyrimidine analogues, we envisioned to use this know-how to determine the regiochemistry of the nucleophilic aromatic substitution on the 2,7-dichlorothiazolo[4,5-*d*]pyrimidine scaffold (as explained in Schemes 1 and 2) through chemical synthesis (Scheme 3). We opted for the simpler substituents on the heterocyclic scaffold, making the interpretation of NMR spectra easier. Therefore, compound **11a** was reacted with ethanol in basic condition, yielding compound **16**. The same compound has been made independently starting from 5-amino-2-bromo-7-ethoxythiazolo[5,4-*d*]pyrimidine **4b.** In this sequence, the ethoxy group is undoubtedly fixed at position 7 (as it is introduced in the first step of the synthesis), and the methoxyethylamine side chain is positioned at position 2 (as has been proven that diazotation preferentially takes place at position 2). As the compounds obtained in both ways were completely identical (NMR, TLC), it indicates that the chlorine at position 2 is more reactive than the one at position 7 and it supports our earlier findings, described in Scheme 2.

3. Biological evaluation

Compounds were assayed in an in vitro colorimetric assay employing recombinant *E. coli* or *S. aureus* SecA, isolated inner membrane vesicles containing overexpressed SecYEG and the *E. coli* preprotein AlkProPhoA(Cys-). The SecA proteins and the AlkProPhoA preprotein have been purified via Ni-NTA-affinity chromatography. The purity of the proteins has been verified via SDS–PAGE and this did not reveal any contaminating proteins (Fig. 2).

Compounds were evaluated at an initial concentration of 200 μM for their ability to inhibit the intrinsic ATPase activity of *E. coli* and *S. aureus* SecA, using the malachite green colorimetric method for the detection of free inorganic phosphate.[38] Because the intrinsic ATPase activity of SecA represents a resting non-active state, compounds were also evaluated for their ability to inhibit the preprotein-stimulated translocation ATPase activity of SecA.

Before setting up these assays, time curves of intrinsic, membrane and translocation ATP hydrolysis by *E. coli* SecA have been generated (Figs. 3 and 4). Under the assay conditions (see 'Section 5'), the intrinsic and membrane ATPase activities are linear up to 60 min, whereas for the translocation ATPase assay, ATP hydrolysis follows a linear time course during the first 30 min of the reaction. Under the experimental conditions used, the intrinsic ATPase activity is about twofold stimulated by *E. coli* membrane vesicles containing overexpressed SecYEG, and approximately 10-fold when also the *E. coli* preproteine AlkProPhoA(Cys-) is present.

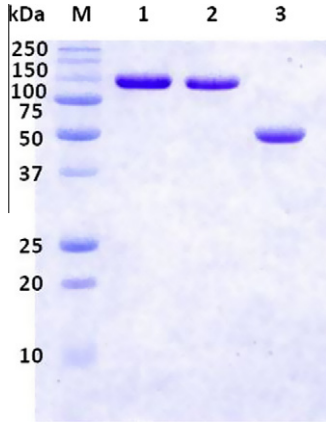


Figure 2. SDS–Polyacrylamide gel electrophoresis of purified recombinant *E. coli* SecA, *S. aureus* SecA1 and the *E. coli* preprotein ProPhoA. Lane M: molecular weight marker; Lane 1: ecSecA; Lane 2: saSecA1; Lane 3: ProPhoA.

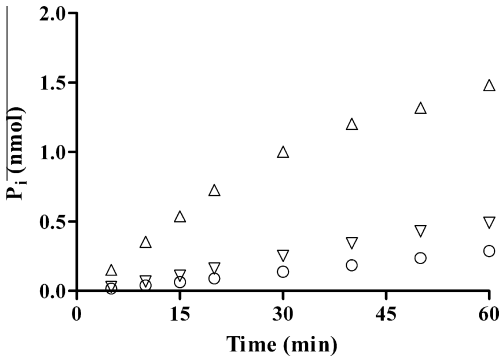


Figure 3. Time courses of intrinsic, membrane and translocation ATP hydrolysis by ecSecA. EcSecA (1 μg/50 μl) was incubated with 100 μM ATP in the presence of IMVs containing overexpressed ecSecYEG and the preprotein ProPhoA (△; translocation ATPase), in the presence of IMVs alone (▽; membrane ATPase) or in the absence of IMVs and ProPhoA (○; intrinsic ATPase). At several time points after the start of the ATPase reaction, released Pi was quantified using the malachite green method as described in 'Section 5'. Data represent the average of three independent experiments, and standard deviations were less <5%.

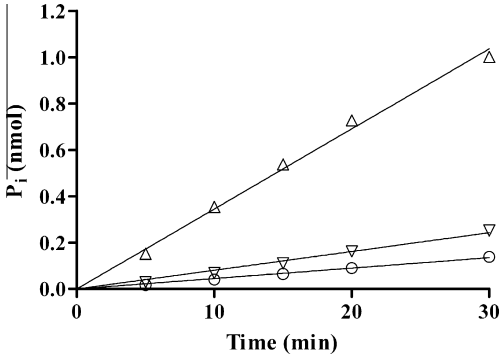


Figure 4. Time courses of intrinsic, membrane and translocation ATP hydrolysis by ecSecA. The linear phase of the progress curves in Figure 3 is shown, indicating that the intrinsic, membrane and translocation ATPase activity of ecSecA is linear during the first 30 min of the reaction.

In order to define the experimental conditions for final screening assays, we have generated time curves of intrinsic ATP hydrolysis at different enzyme concentrations. As can be derived from Figure 5, the time course of intrinsic *E. coli* SecA ATPase activity is linear up to 120 min for *E. coli* SecA concentrations between 0.5 and 2.5 μg ecSecA/50 μl. Based on these results, we have defined a fixed *E. coli* SecA concentration of 2.5 μg/50 μl and an incubation time of 2 h for screening assays. As the hydrolysis of ATP under these experimental conditions is linear up to 120 min, the rate of ATP hydrolysis can be determined by dividing the amount of Pi formed by 120 (yielding nmol Pi/min). Similar experiments have been performed for *S. aureus* SecA (Fig. 6) from which the final conditions for the *S. aureus* SecA screening assay have been established (28 °C, 20 μg saSecA1/50 μl, 2 h incubation time).

Compounds **5i**, **7b**, **12a**, **12b** and **14a** display around 50% inhibition of the intrinsic *E. coli* SecA ATPase activity at 200 μM (Fig. 7). They all have either a fluorophenyl or a piperazine substituent at position 7 of the scaffold. Two compounds (**5b** and **14b**) display more pronounced *E. coli* SecA inhibition, with 70% and 76% inhibition, respectively. These compounds were selected for dose–responses titration curves, yielding IC_{50} values of 197 and 135 μM, respectively (Fig. 8).

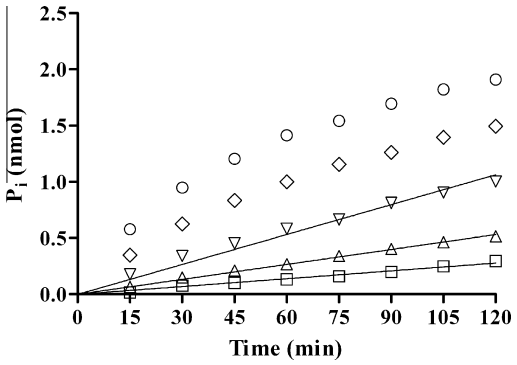


Figure 5. Time courses of intrinsic ATP hydrolysis by ecSecA. EcSecA (0.5–10 μg/50 μl) was incubated at 37 °C with 100 μM ATP in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, pH 8.0). At several time points after the start of the ATPase reaction, released Pi was quantified using the malachite green method as described in experimental section. Data represent the average of three independent experiments, and standard deviations were less <5%. Symbols used: □ 0.5 μg ecSecA/50 μl; △ 1 μg ecSecA/50 μl; ▽ 2.5 μg ecSecA/50 μl; ◇ 5 μg ecSecA/50 μl; ○ 10 μg ecSecA/50 μl.

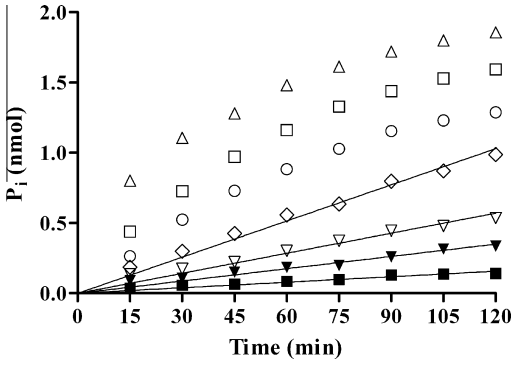


Figure 6. Time courses of intrinsic ATP hydrolysis by saSecA1. saSecA1 (2.5–50 μg/50 μl) was incubated at 28 °C with 100 μM ATP in 50 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, pH 7.5). At several time points after the start of the ATPase reaction, released Pi was quantified using the malachite green method as described in Materials and Methods. Data represent the average of three independent experiments, and standard deviations were less <5%. Symbols used: ■ 2.5 μg saSecA1/50 μl; ▼5 μg saSecA1/50 μl; ▽ 10 μg saSecA1/50 μl; ◇ 20 μg saSecA1/50 μl; ○ 30 μg saSecA1/50 μl; □ 40 μg saSecA1/50 μl; △ 50 μg saSecA1/50 μl.

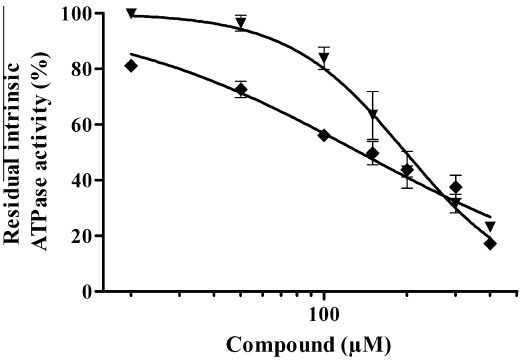


Figure 8. Inhibition of the intrinsic ATPase activity of ecSecA by compound **5b** and **14b**. Initial rates of intrinsic ATP hydrolysis by ecSecA were measured at 37 °C in the presence of 100 μM ATP and various concentrations (0–200 μM) of compound **5b** (▼) and **14b** (♦). Rates were expressed relative to the rate of ATP hydrolysis measured in the absence of compound. IC_{50} values were determined by fitting the data by nonlinear regression analysis using GraphPad Prism. Values are the means of three independent experiments, with error bars representing the standard deviation.

By comparing the biological activity of compounds **12b**, **13**, **14a** and **14b**, it is evident that by subtle structural variation of the piperazine moiety the biological activity can be influenced. The free piperazine moiety (compound **13**) affords 35% inhibition, whereas derivatisation as a carbamate (compound **12b**) or a urea (compound **14a**) furnished compounds displaying 50% inhibition. Reaction of the piperazine with an acyl chloride affords an amide congener **14b** with an IC_{50} value of 135 μM. In the 7-*N*-morpholino series, the nature of the substituent at position 2 seems important for *E. coli* SecA inhibition. The styryl group (compound **5b**) is the most potent compound (70% inhibition at 200 μM), whereas other substituents, such as alkylamino groups (compounds **5e** and **5f**), phenyl (compounds **5a** and **5c**) or acetylene moiety (compound **5d**) are less active. The fact that compound **5c** (which is structurally very related to compound **5b)** is totally devoid of SecA inhibitory activity, points towards a specific inhibitory activity of the compounds.

In general, the compounds were less efficient in inhibiting the translocation *E. coli* SecA ATPase activity when compared to their effect on the intrinsic ATPase activity of *E. coli* SecA. However, the two most potent analogues (compounds **5b** and **14b**) had also

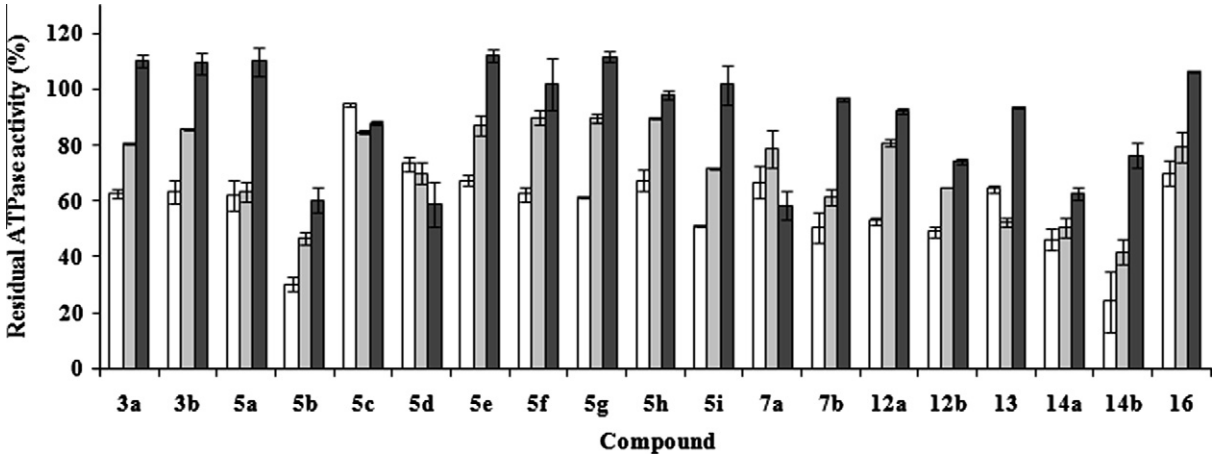


Figure 7. Inhibition of the intrinsic ATPase activity of saSecA and ecSecA and of the translocation ATPase activity of ecSecA by different thiazolo[4,5-*d*]pyrimidines. Initial rates of intrinsic ATP hydrolysis by saSecA (dark grey bars) and ecSecA (white bars) and the translocation ATPase activity of ecSecA (light grey bars) were measured in the presence of 100 μM ATP and 200 μM compound. Rates were expressed relative to the rate of ATP hydrolysis measured in the absence of compound. Values are the means of three independent experiments, with error bars representing the standard deviation.

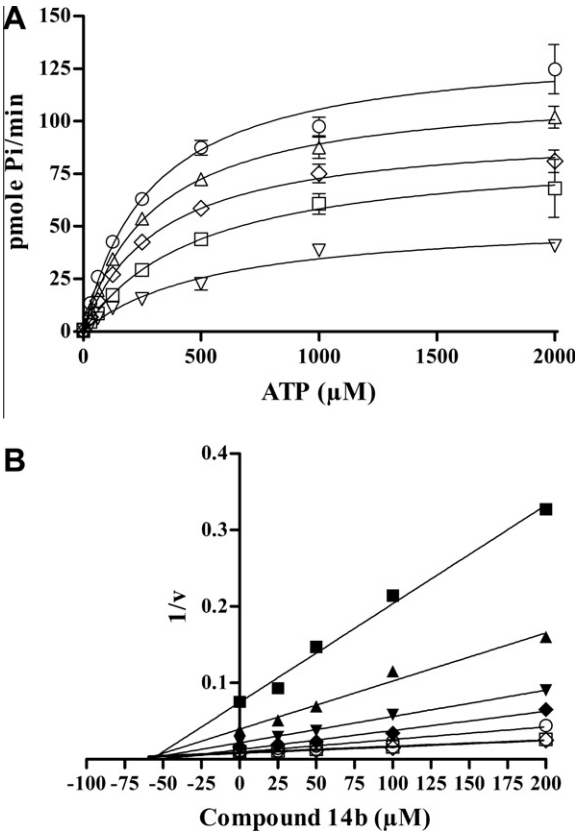


Figure 9. Inhibition of the ecSecA translocation ATPase activity by compound **14b**. (A) Initial rates of translocation ATP hydrolysis by ecSecA were measured in the presence of different ATP (0–2 mM) and compound concentrations (0–200 μM), as described in 'Section 5'. Apparent K_m and V_{max} values were determined by fitting the data by nonlinear regression analysis to the Michaelis–Menten equation. Data represent the average of three independent experiments, with error bars representing the standard deviation. Symbols used: ○ no inhibitor; △ 25 μM inhibitor; ◇ 50 μM inhibitor; □ 100 μM inhibitor; ▽ 200 μM inhibitor. (B) Dixon plot of the data in Figure 4A. Linear regression of the data yielded a K_i value of 60 μM for inhibition of the ecSecA translocation ATPase. Symbols used: ■ 5 μM ATP; ▲ 10 μM ATP; ▼50 μM ATP; ♦ 100 μM ATP; ○ 500 μM ATP; □ 1000 μM ATP; ◇ 2000 μM ATP.

pronounced effect on the translocation ATPase activity of SecA (more than 50% inhibition at 200 μM).

The most promising analogue (compound **14b**) was selected for K_i-determination. Initial rates of ATP hydrolysis were measured in the *E. coli* SecA translocation ATPase activity assay at different ATP (0–2 mM) and inhibitor (0–200 μM) concentrations (Fig. 9A). Data were fit by nonlinear regression analysis to the Michaelis–Menten equation and the apparent K_m and V_{max} values were determined for each inhibitor concentration (Table 2). It is clear that compound **14b** increases K_m and decreases V_{max}, which is indicative of 'mixed-type' inhibition. In other words, inhibition of the ecSecA translocation ATPase activity by compound **14b** has both a non-competitive and a competitive component, resulting from a combination of a decreased turnover number and decreased affinity of the active site for ATP. Dixon plot analysis yielded a K_i value of 60 μM for inhibition of the SecA translocation ATPase activity (Fig. 9B).

Table 2
Apparent K_m and V_{max} values for the ecSecA translocation ATPase activity in the presence of different concentrations of compound **14b**

	μM Inhibitor				
	0	25	50	100	200
$V_{max,app}$	136.1	115.8	96.1	86.5	54.2
$K_{m,app}$	288.1	298.4	319.8	484.0	567.4

Overall, it seems that the compounds are less active against SecA of *S. aureus* than of *E. coli*. An explanation for this observation cannot be provided at this moment however, but local structural differences in the compound binding site of *E. coli* and *S. aureus* SecA might explain this apparent discrepancy. At a concentration of 200 μM, the most potent compounds (**5b**, **5d**, **7a** and **14a**) display around 40% inhibition of the *S. aureus* SecA ATPase activity. To the best of our knowledge, these compounds represent the first examples of *S. aureus* SecA inhibitors.

Although the activity is weak, they can be used as starting points for further optimization. The chemistry described in this paper will enable to rapidly explore the SAR with respect to SecA inhibition and to improve the potency. In addition, compound **5b** has a molecular weight of only 339 and can be considered as a fragment, rather than a hit. Hence, fragment based drug discovery could be employed as the X-ray crystal structure of SecA from *E. coli* is available.[14]

4. Conclusion

In summary, the investigation of regioselective reactions on a 2,7-dichlorothiazolo[4,5-*d*]pyrimidine scaffold has shown that the chlorine at position 2 is more reactive than the one at position 7. In addition, regioselective diazotations on a 2,5-diamino-thiazolo[4,5-*d*]pyrimidine leads selectively to the 2-halo-5-amino-thiazolo[4,5-*d*]pyrimidine. These findings can be exploited for the sequential introduction of substituents at positions 2 and 7 and allow for the construction of a highly diverse 5-amino-thiazolo[4,5-*d*]pyrimidine library. Screening of this compound library against SecA led to the discovery of hits with activity against SecA from *E. coli* and *S. aureus*. Currently, we are exploiting this chemistry in order to improve the biological activity of the hits.

5. Experimental section

5.1. General

For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glass-ware (135 °C). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 300 (^{1}H NMR: 300 MHz, ^{13}C NMR: 75 MHz), using tetramethylsilane as internal standard for ^{1}H NMR spectra and DMSO-d_6 (39.5 ppm) or $CDCl_3$ (77.2 ppm) for ^{13}C NMR spectra. Abbreviations used are: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet. Coupling constants are expressed in Hertz. Mass spectra are obtained with a Finnigan LCQ advantage Max (ion trap) mass spectrophotometer from Thermo Finnigan, San Jose, CA, USA. Exact mass measurements are performed on a quadrupole time-of-flight mass spectrometer (Q-tof-2, Micromass, Manchester, UK) equipped with a standard electrospray-ionization (ESI) interface. Samples were infused in *i*-PrOH/H_2O (1:1) at 3 μl/min. Melting points are determined on a Barnstead IA 9200 and are uncorrected. Precoated aluminum sheets (Fluka Silica gel/TLC-cards, 254 nm) were used for TLC. Column chromatography was performed on ICN silica gel 63–200, 60 Å.

5.2. General procedure for the preparation of compounds 1–16

5.2.1. 6-Morpholinopyrimidine-2,4-diamine (1a)

To a suspension of 2,6-diamino-4-chloropyrimidine (3.0 g, 20.7 mmol) in water (100 ml) was added morpholine (7.26 ml, 83.0 mmol). The mixture was refluxed overnight. After cooling down to room temperature, the solution was treated with a 1 N

NaOH solution to lead a precipitate. The resulting solid was filtered off, washed with water and dried over P_2O_5, furnishing the title compound as a pale yellow solid (3.3 g, 81%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 5.78 (s, 2H, NH_2), 5.59 (s, 2H, NH_2), 5.03 (s, 1H, CH), 3.60 (br s, 4H, $O(CH_2)_2$), 3.31 (br s, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 165.2, 164.1, 162.6, 74.1, 65.9, 44.2 ppm. MS: 196.06 $[M+H]^+$

5.2.2. 6-Ethoxypyrimidine-2,4-diamine (1b)

To a solution of NaH (2.77 g, 69.2 mmol) in ethanol (150 ml) was added 2,6-diamino-4-chloropyrimidine (5.0 g, 34.6 mmol). The mixture was refluxed for 6 h. After cooling, the solution was neutralized with a 5–6 N HCl solution in isopropyl alcohol. After removing the solvents in vacuo, the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 40:1), yielding the title compound as a white solid (4.0 g, 75%). Mp 164–165 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 6.00 (s, 2H, NH_2), 5.88 (s, 2H, NH_2), 5.03 (s, 1H, CH), 4.13 (q, J = 7.1 Hz, 2H, CH_2), 1.22 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 170.0, 165.9, 162.9, 76.1, 60.2, 14.7 ppm. HRMS: calcd for $C_6H_{11}N_4O$ $[M+H]^+$ 155.09329, found 155.09260.

5.2.3. 6-(Isopentyloxy)pyrimidine-2,4-diamine (1c)

This compound was synthesized according to the procedure for the preparation of compound **1b**, using isoamyl alcohol. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a pale yellow oil (95%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 5.23 (s, 1H, C═CH), 4.69 (s, 2H, NH_2), 4.51 (s, 2H, NH_2), 4.18 (t, J = 6.7 Hz, 2H, OCH_2), 1.77 (m, 1H, CH), 1.60 (q, J = 6.7 Hz, 2H, CH_2), 0.94 (d, J = 6.6 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 170.6, 165.3, 162.3, 76.6, 63.8, 37.1, 24.3, 21.9 ppm. HRMS: calcd for $C_9H_{17}N_4O$ $[M+H]^+$ 197.14024, found 197.13955.

5.2.4. 1-(4-(2,6-Diaminopyrimidin-4-yl)piperazin-1-yl) ethanone (1d)

To a solution of 2,6-diamino-4-chloropyrimidine (3.0 g, 20.8 mmol) in water (100 ml) was added *N*-acetylpiperazine (10.64 g, 83.0 mmol). The mixture was refluxed for 21 h. The orange solution was cooled down and made alkaline with a 10 M NaOH solution. The white precipitate was filtered off, washed with cold water and dried over P_2O_5 in a vacuum desiccator to yield the title compound as a white solid (3.3 g, 67%). Mp 274 °C ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 5.74 (s, 2H, NH_2), 5.51 (s, 2H, NH_2), 5.04 (s, 1H, CH), 3.33–3.46 (m, 8H, $N(CH_2)_2$, $CON(CH_2)_2$), 2.02 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 168.3, 165.2, 163.5, 162.7, 74.1, 45.2, 43.7, 43.3, 40.5, 21.2 ppm. HRMS: calcd for $C_{10}H_{17}N_6O$ $[M+H]^+$ 237.1464, found 237.1454.

5.2.5. 6-Morpholino-5-thiocyanatopyrimidine-2,4-diamine (2a)

A solution of 6-morpholinopyrimidine-2,4-diamine **1a** (5.0 g, 25.6 mmol) and potassium thiocyanate (14.9 g, 0.15 mol) in DMF (120 ml) was heated at 65 °C. Pyridine (4.14 ml, 51.2 mmol) was added and the solution cooled to 5 °C. Bromine (1.31 ml, 25.6 mmol) was added slowly and the reaction mixture stirred for 2 h at 5–10 °C. The reaction mixture was poured into ice-water (100 ml) and stirred for 1 h. The volatiles were removed under reduced pressure and the resulting solid was diluted with water, filtered and dried. The crude solid was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 30:1) to yield the title compound as white solid (3.61 g, 56%). Mp 161 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 6.78 (s, 2H, NH_2), 6.36 (s, 2H, NH_2), 3.69 (br s, 4H, $O(CH_2)_2$), 3.41 (br s, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 168.1, 166.5, 162.6, 112.3, 69.1, 66.1, 49.2 ppm. HRMS: calcd for $C_9H_{13}N_6OS$ $[M+H]^+$ 253.08715, found 253.08637.

5.2.6. 6-Ethoxy-5-thiocyanatopyrimidine-2,4-diamine (2b)

This compound was synthesized from **1b** according to the procedure for the preparation of compound **2a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a pale yellow solid (89%). Mp 182 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 6.88 (s, 2H, NH_2), 6.61 (s, 2H, NH_2), 4.31 (q, J = 7.1 Hz, 2H, CH_2), 1.30 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 169.1, 165.6, 163.1, 112.1, 66.5, 62.0, 14.6 ppm. HRMS: calcd for $C_7H_{10}N_5OS$ $[M+H]^+$ 212.06061, found 212.05973.

5.2.7. 6-(Isopentyloxy)-5-thiocyanatopyrimidine-2,4-diamine (2c)

This compound was synthesized from **1c**, according to the procedure for the preparation of compound **2a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a pale yellow solid (76%). Mp 152 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 6.87 (s, 2H, NH_2), 6.59 (s, 2H, NH_2), 4.29 (t, J = 6.6 Hz, 2H, OCH_2), 1.74 (sixtet, J = 6.6 Hz, 1H, CH), 1.59 (q, J = 6.7 Hz, 2H, CH_2), 0.93 (d, J = 6.6 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 169.1, 165.6, 163.1, 112.1, 66.6, 64.6, 37.2, 24.6, 22.4 ppm. HRMS: calcd for $C_{10}H_{16}N_5OS$ $[M+H]^+$ 254.10756, found 254.10689.

5.2.8. 7-Morpholinothiazolo[4,5-*d*]pyrimidine-2,5-diamine (3a)

6-Morpholino-5-thiocyanatopyrimidine-2,4-diamine **2a** (3.5 g, 14.3 mmol) was heated at 140 °C in a DMF/water solution (4:1, 50 ml) for two days. After cooling, the resulting solid was filtered off, washed with water and ethyl acetate, furnishing the title compound as a yellow solid (2.1 g, 58%). Mp 242 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 7.95 (s, 2H, NH_2), 6.01 (s, 2H, NH_2), 3.66 (br s, 4H, $O(CH_2)_2$), 3.58 (br s, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 171.2, 170.6, 160.9, 157.8, 90.8, 65.9, 45.7 ppm. HRMS: calcd for $C_9H_{13}N_6OS$ $[M+H]^+$ 253.08715, found 253.08621.

5.2.9. 7-Ethoxythiazolo[4,5-*d*]pyrimidine-2,5-diamine (3b)

This compound was synthesized from **2b** according to the procedure for the preparation of compound **3a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 20:1) to yield the title compound as a pale yellow solid (76%). Mp 224 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.05 (s, 2H, NH_2), 6.28 (s, 2H, NH_2), 4.37 (q, J = 7.1 Hz, 2H, CH_2), 1.30 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 172.7, 171.6, 162.6, 162.2, 92.4, 61.4, 14.6 ppm. HRMS: calcd for $C_7H_{10}N_5OS$ $[M+H]^+$ 12.06061, found 212.05993.

5.2.10. 7-(Isopentyloxy)thiazolo[4,5-*d*]pyrimidine-2,5-diamine (3c)

This compound was synthesized from **2c** in a yield of 91%, according to a procedure for the preparation of compound **3a**. Mp 212 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.06 (s, 2H, NH_2), 6.30 (s, 2H, NH_2), 4.35 (t, J = 6.7 Hz, 2H, OCH_2), 1.69 (sixtet, J = 6.5 Hz, 1H, CH), 1.59 (q, J = 6.6 Hz, 2H, CH_2), 0.92 (d, J = 6.5 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 172.5, 171.6, 162.7, 162.1, 92.4, 64.1, 37.2, 24.7, 22.4 ppm. HRMS: calcd for $C_{10}H_{16}N_5OS$ $[M+H]^+$ 254.10756, found 254.10675.

5.2.11. 1-(4-(2,5-Diaminothiazolo[4,5-*d*]pyrimidin-7-yl) piperazin-1-yl)ethanone (3d)

This compound was synthesized from **1d** according to the procedure for the preparation of compound **2a**. The crude residue was triturated with hot methanol to yield the title compound as a pale yellow solid (52%). Mp 263 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 7.85 (s, 2H, NH_2), 5.90 (s, 2H, NH_2), 3.53–3.64 (m, 8H, $N(CH_2)_2$, $CON(CH_2)_2$), 2.03 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.2, 170.2, 168.5, 161.6, 157.5, 90.9, 45.3, 45.2,

44.9, 40.6, 21.2 ppm. HRMS: calcd for $C_{11}H_{16}N_7OS$ $[M+H]^+$ 294.1137, found 294.1111.

5.2.12. 2-Bromo-7-morpholinothiazolo[4,5-*d*]pyrimidin-5-amine (4a)

To a solution of 7-morpholinothiazolo[4,5-*d*]pyrimidine-2,5-diamine **3a** (1.0 g, 3.96 mmol) in acetonitrile (25 ml) were added bromotrimethylsilane (1.57 ml, 11.9 mmol) and isoamyl nitrite (0.67 ml, 4.76 mmol). The mixture was stirred at room temperature for two days. After removing the solvents, the crude residue was extracted with dichloromethane, brine and dried over Na_2SO_4. After evaporating the solvents, the crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 50:1) to yield the title compound as a pale yellow solid (0.69 g, 55%). Mp 253 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 4.85 (s, 2H, NH_2), 3.79 (s, 8H, $O(CH_2)_2$, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 170.8, 161.8, 158.6, 102.0, 66.7, 46.1 ppm. HRMS: calcd for $C_9H_{11}BrN_5OS$ $[M+H]^+$ 315.98677/317.98472, found 315.98560/317.98353.

5.2.13. 2-Bromo-7-ethoxythiazolo[4,5-*d*]pyrimidin-5-amine (4b)

This compound was synthesized from **3b** according to the procedure for the preparation of compound **4a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 80:1) to yield the title compound as a pale yellow solid (48%). Mp 203 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 5.06 (s, 2H, NH_2), 4.51 (q, J = 7.1 Hz, 2H, CH_2), 1.42 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 170.7, 163.2, 162.9, 146.2, 101.8, 62.8, 14.2 ppm. HRMS: calcd for $C_7H_8BrN_4OS$ $[M+H]^+$ 274.96022/276.95817, found 274.95958/276.95757.

5.2.14. 2-Bromo-7-(isopentyloxy)thiazolo[4,5-*d*]pyrimidin-5-amine (4c)

This compound was synthesized from **3c,** according to the procedure for the preparation of compound **4a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a pale yellow solid (0.19 g, 80%). Mp 181–182 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 5.32 (s, 2H, NH_2), 4.47 (t, J = 6.7 Hz, 2H, OCH_2), 1.73 (sixtet, J = 6.3 Hz, 1H, CH), 1.69 (q, J = 6.5 Hz, 2H, CH_2), 0.97 (d, J = 6.4 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 170.8, 164.5, 162.4, 147.0, 105.1, 66.3, 37.5, 25.3, 22.7 ppm MS: calcd for $C_{10}H_{14}BrN_4OS$ $[M+H]^+$ 317.01, 319.01, found 316.88, 318.91.

5.2.15. 1-(4-(5-Amino-2-chlorothiazolo[4,5-*d*]pyrimidin-7-yl) piperazin-1-yl)ethanone (4d)

A solution of $NaNO_2$ (94 mg, 1.36 mmol) in H_2SO_4 (1.7 ml) was heated at 80 °C until the solid dissolved. The solution was cooled down to room temperature and a solution of 1-(4-(2,5-diaminothiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone **3d** (0.1 g, 0.34 mmol) in acetic acid (0.5 ml) was added. After the addition was completed, the solution was stirred at 40 °C for 30 min. A solution of CuCl (0.1 g, 1.02 mmol) in HCl (0.5 ml) was added and the mixture was heated at 80 °C for 30 min. After cooling, water (3 ml) was added. The mixture was neutralized with a 10 N NaOH solution and extracted with a $CHCl_3$/EtOH (4/1) solution, brine and dried over Na_2SO_4. After removing the solvents under reduced pressure, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 20:1) affording the title compound as a white solid (65 mg, 61%). Mp 230 °C decomposed ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 6.46 (s, 2H, NH_2), 3.71–3.80 (m, 4H, $N(CH_2)_2$), 3.56–3.61 (m, 4H, $CON(CH_2)_2$), 2.04 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 169.3, 168.6, 162.1, 158.1, 157.8, 86.1, 44.9, 44.8, 44.6, 40.4, 21.2 ppm. HRMS: calcd for $C_{11}H_{14}ClN_6OS$ $[M+H]^+$ 313.0638, found 313.0622.

5.2.16. 2-(4-Fluorophenyl)-7-morpholinothiazolo[4,5-*d*] pyrimidin-5-amine (5a)

To a solution of 2-bromo-7-morpholinothiazolo[4,5-*d*]pyrimidin-5-amine **4a** (0.10 g, 0.32 mmol) in dioxane/water (3:1, 4 ml), was added 4-fluorophenylboronic acid (53 mg, 0.38 mmol), K_2CO_3 (0.18 g, 1.27 mmol) and $Pd(PPh_3)_4$ (37 mg, 0.03 mmol). The reaction mixture was refluxed under N_2 overnight. After cooling down to room temperature, 1 N HCl was added slowly to neutralize the mixture to pH 7–8. The mixture was extracted with dichloromethane, brine and dried over Na_2SO_4. After removing the solvent, the residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 70:1) to yield the title compound as a pale yellow solid (92.4 mg, 88%). Mp 296 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.09–8.14 (m, 2H, ArH), 7.17 (t, J = 8.5 Hz, 2H, ArH), 4.82 (s, 2H, NH_2), 3.88 (s, 4H, $O(CH_2)_2$), 3.84 (s, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.9, 170.9, 165.2 (d, J = 251.6 Hz), 161.9, 159.5, 129.6 (d, J = 8.8 Hz), 129.2 (d, J = 3.3 Hz), 116.5 (d, J = 22.0 Hz), 99.8, 66.9, 46.3 ppm. HRMS: calcd for $C_{15}H_{15}FN_5OS$ $[M+H]^+$ 332.09813, found 332.09694.

5.2.17. (*E*)-7-Morpholino-2-styrylthiazolo[4,5-*d*]pyrimidin-5-amine (5b)

This compound was synthesized according to a procedure for the preparation of compound **5a**, using (*E*)-styrylboronic acid. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 70:1) to yield the title compound as a pale yellow solid (32%). Mp 255 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.74 (d, J = 16.0 Hz, 1H, CH), 7.58 (d, J = 8.0 Hz, 2H, ArH), 7.39–7.43 (m, 3H, ArH), 7.26 (d, J = 16.0 Hz, 1H, CH), 4.81 (s, 2H, NH_2), 3.86 (s, 4H, $O(CH_2)_2$), 3.83 (s, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.7, 170.6, 161.9, 159.4, 138.4, 135.2, 130.0, 129.1, 127.9, 124.0, 120.8, 66.9, 46.3 ppm. HRMS: calcd for $C_{17}H_{18}N_5OS$ $[M+H]^+$ 340.12321, found 340.12186.

5.2.18. 7-Morpholino-2-phenylthiazolo[4,5-*d*]pyrimidin-5-amine (5c)

To a mixture of $Pd(PPh_3)_4$ (15 mg, 0.01 mmol) and 2-bromo-7-morpholinothiazolo[4,5-*d*]pyrimidin-5-amine **4a** (40 mg, 0.13 mmol) in dioxane (1 ml) was added tributylphenylstannane (45 μl, 0.14 mmol). The reaction mixture was refluxed overnight under a nitrogen atmosphere and then cooled down to room temperature. The solvents were removed under reduced pressure. The crude residue was diluted with dichloromethane and washed with water. The combined organic layers were dried over Na_2SO_4 and the solvent was evaporated in vacuo. The crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 60:1), yielding the pure title compound as a yellow solid (20 mg, 50%). Mp 278 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.11 (d, J = 7.8 Hz, 2H, ArH), 7.50–7.44 (m, 3H, ArH), 4.81 (s, 2H, NH_2), 3.88–3.91 (m, 4H, $O(CH_2)_2$), 3.82–3.85 (m, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.9, 172.3, 161.9, 159.5, 132.9, 132.0, 129.2, 127.5, 110.9, 66.9, 46.3 ppm. HRMS: calcd for $C_{15}H_{16}N_5OS$ $[M+H]^+$ 314.10756, found 314.10635.

5.2.19. 7-Morpholino-2-(phenylethynyl)thiazolo[4,5-*d*]pyrimidin-5-amine (5d)

To a mixture of bis(triphenylphosphine)palladium(II) acetate (2 mg, 0.002 mmol), 2-bromo-7-morpholinothiazolo[4,5-*d*]pyrimidin-5-amine **4a** (40 mg, 0.13 mmol) and triethylamine (63 μl) in dioxane (2 ml) was added a solution of phenylacetylene (20 μl, 0.14 mmol) in DMF (0.3 ml) over a period of 5 min. The reaction mixture was refluxed under nitrogen for 6 h and then cooled to room temperature. The solvents were removed under reduced pressure. The crude residue was diluted with dichloromethane and washed with water. The combined organic layers were dried over $MgSO_4$ and evaporated in vacuo. The crude residue was

purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 60:1), yielding the pure title compound as an orange solid (38 mg, 89%). Mp >260 °C decomposed ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.62 (d, J = 7.7 Hz, 2H, ArH), 7.38–7.48 (m, 3H, ArH), 5.00 (s, 2H, NH_2), 3.84 (s, 4H, $O(CH_2)_2$), 3.82 (s, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.0, 162.3, 159.2, 153.1, 132.4, 130.4, 128.8, 120.9, 100.3, 98.3, 82.6, 66.8, 46.3 ppm. HRMS: calcd for $C_{17}H_{16}N_5OS$ $[M+H]^+$ 338.10756, found 338.10645.

5.2.20. 2,7-Dimorpholinothiazolo[4,5-*d*]pyrimidin-5-amine (5e)

A solution of 2-bromo-7-morpholinothiazolo[4,5-*d*]pyrimidin-5-amine **4a** (40 mg, 0.13 mmol) in morpholine (2 ml) was heated at 80 °C under a nitrogen atmosphere overnight and then cooled to room temperature. The volatiles were removed under reduced pressure. The crude residue was diluted with dichloromethane and washed with water. The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated in vacuo. The crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 40:1) to yield the title compound as white solid (35 mg, 86%). Mp 251–253 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.03 (s, 2H, NH_2), 2.72 (s, 8H, $O(CH_2)_2$), 2.66 (s, 8H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 171.9, 171.8, 161.5, 158.5, 92.9, 66.9, 66.2, 48.2, 46.2 ppm. HRMS: calcd for $C_{13}H_{19}N_6O_2S$ $[M+H]^+$ 323.12902, found 323.12811.

5.2.21. *N*-2-(2-Methoxyethyl)-7-morpholinothiazolo[4,5-*d*]pyrimidine-2,5-diamine (5f)

This compound was synthesized according to a procedure for the preparation of compound **5e**, using 2-methoxyethylamine. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a white solid (93%). Mp 205–206 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 5.94 (s, 1H, NH), 4.67 (s, 2H, NH_2), 3.75–3.79 (m, 4H, $O(CH_2)_2$), 3.61–3.71 (m, 6H, CH_2, $N(CH_2)_2$), 3.62 (t, J = 5.0 Hz, 2H, CH_2), 3.39 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 171.8, 170.9, 161.4, 158.6, 128.9, 70.7, 66.9, 59.0, 46.2, 44.7 ppm. HRMS: calcd for $C_{12}H_{19}N_6O_2S$ $[M+H]^+$ 11.12902, found 311.12837.

5.2.22. 7-Ethoxy-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-5-amine (5g)

This compound was synthesized from **4b** according to the procedure for the preparation of compound **5a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 200:1) to yield the title compound as a white solid (76%). Mp 194 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.09–8.14 (m, 2H, ArH), 7.17 (t, J = 8.5 Hz, 2H, ArH), 5.08 (s, 2H, NH_2), 4.54 (q, J = 7.1 Hz, 2H, OCH_2), 1.45 (t, J = 7.1 Hz, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 173.3, 172.7, 165.4, 165.2 (d, J = 251.7 Hz), 162.3, 129.8 (d, J = 8.8 Hz), 129.5 (d, J = 3.3 Hz), 116.4 (d, J = 22.0 Hz), 63.4, 14.6 ppm. HRMS: calcd for $C_{13}H_{12}FN_4OS$ $[M+H]^+$ 291.07158, found 291.07079.

5.2.23. 2-(4-Fluorophenyl)-7-(isopentyloxy)thiazolo[4,5-*d*]pyrimidin-5-amine (5h)

This compound was synthesized from **4c** according to the procedure for the preparation of compound **5a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 70:1) to yield the title compound as a pale yellow solid (86%). Mp 210 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.10–8.15 (m, 2H, ArH), 7.17 (t, J = 8.5 Hz, 2H, ArH), 5.01 (s, 2H, NH_2), 4.51 (t, J = 6.8 Hz, 2H, OCH_2), 1.81 (sixtet, J = 6.4 Hz, 1H, CH), 1.72 (q, J = 6.5 Hz, 2H, CH_2), 0.99 (d, J = 6.5 Hz, 6H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 173.2, 172.7, 165.5, 165.2 (d, J = 251.6 Hz), 162.4, 129.8 (d, J = 8.8 Hz), 129.6 (d, J = 3.3 Hz), 116.4 (d, J = 22.0 Hz), 114.2, 66.1, 37.6, 25.4, 22.8 ppm. HRMS: calcd for $C_{16}H_{18}FN_4OS$ $[M+H]^+$ 333.11854, found 333.11794.

5.2.24. 1-(4-(5-Amino-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone (5i)

This compound was synthesized from **4d**, according to the procedure for the preparation of compound **5a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 30:1) to yield the title compound as a yellow solid (73%). Mp 280 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.09–8.14 (m, 2H, PhH), 7.42 (t, J = 8.8 Hz, 2H, PhH), 6.33 (s, 2H, NH_2), 3.88 (br s, 2H, NCH_2), 3.81 (br s, 2H, NCH_2), 3.61 (br s, 4H, $CON(CH_2)_2$), 2.06 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.4, 169.4, 168.6, 164.2 (d, J = 248.7 Hz), 162.2, 158.5, 129.2 (d, J = 9.0 Hz), 128.9 (d, J = 2.9 Hz), 116.6 (d, J = 22.1 Hz), 97.5, 45.1, 44.8, 40.5, 40.3, 21.2 ppm. HRMS: calcd for $C_{17}H_{18}FN_6OS$ $[M+H]^+$ 373.1247, found 373.1223.

5.2.25. 2-(4-Fluorophenyl)-7-(piperazin-1-yl)thiazolo[4,5-*d*]pyrimidin-5-amine (6)

A solution of 1-(4-(5-amino-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)ethanone **5i** (0.1 g, 0.27 mmol) in 5% HCl (2 ml) was heated at 100 °C for 4 h. After cooling down to room temperature, the mixture was neutralized with 2 N NaOH to pH 7. The precipitate was filtered off, washed with water and dried. The crude compound was purified by flash chromatography on silica (CH_2Cl_2/MeOH 5:1) to yield the title compound as a yellow solid (81 mg, 91%). Mp 260 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.10–8.15 (m, 2H, PhH), 7.41 (t, J = 8.8 Hz, 2H, PhH), 6.26 (s, 2H, NH_2), 3.76 (br s, 4H, $N(CH_2)_2$), 2.82 (br s, 4H, $NH(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.4, 169.1, 164.2 (d, J = 248.6 Hz), 162.2, 158.6, 129.2 (d, J = 9.0 Hz), 128.9 (d, J = 3.1 Hz), 116.5 (d, J = 22.1 Hz), 97.3, 46.5, 45.4 ppm. HRMS: calcd for $C_{15}H_{16}FN_6S$ $[M+H]^+$ 331.11412, found 331.11335.

5.2.26. 4-(5-Amino-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-7-yl)-*N*-*m*-tolylpiperazine-1-carboxamide (7a)

To a solution of 2-(4-fluorophenyl)-7-(piperazin-1-yl)thiazolo[4,5-*d*]pyrimidin-5-amine **6** (30 mg, 0.09 mmol) in DMF (1 ml) was added *m*-tolyl isocyanate (13 μl, 0.10 mmol) in DMF (0.3 ml). The reaction mixture was stirred for 2 h at room temperature. The reaction was quenched with water and extracted with EtOAc. The combined organic layers were dried over Na_2SO_4. After removing the solvents in vacuo, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 100:1) to yield the title compound as a white solid (40 mg, 95%). Mp 232–233 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.54 (s, 1H, NH), 8.11–8.16 (m, 2H, PhH), 7.43 (t, J = 8.7 Hz, 2H, PhH), 7.31 (s, 1H, PhH), 7.28 (d, J = 7.5 Hz, 1H, PhH), 7.15 (t, J = 7.5 Hz, 1H, PhH), 6.77 (d, J = 7.5 Hz, 1H, PhH), 6.33 (s, 2H, NH_2), 3.89 (br s, 4H, $N(CH_2)_2$), 3.63 (br s, 4H, $CON(CH_2)_2$), 2.26 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.4, 169.4, 164.2 (d, J = 248.8 Hz), 162.2, 158.6, 154.9, 140.2, 137.3, 129.2 (d, J = 8.9 Hz), 128.9 (d, J = 2.9 Hz), 128.2, 122.6, 120.3, 116.8, 116.5 (d, J = 22.1 Hz), 97.6, 44.9, 43.3, 21.2 ppm. HRMS: calcd for $C_{23}H_{23}FN_7OS$ $[M+H]^+$ 464.16688, found 464.16510.

5.2.27. 1-(4-(5-Amino-2-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-7-yl)piperazin-1-yl)-2-(4-chlorophenoxy)ethanone (7b)

To a solution of 2-(4-fluorophenyl)-7-(piperazin-1-yl)thiazolo[4,5-*d*]pyrimidin-5-amine **6** (30 mg, 0.09 mmol) and pyridine (11 μl, 0.14 mmol) in DMF (1 ml) was added 4-chlorophenoxyacetyl chloride (20 mg, 0.10 mmol). The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was quenched with water, extracted with EtOAc, brine and was dried over Na_2SO_4. After removing the solvents, the crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 100:1) to

yield the title compound as a yellow solid (40 mg, 88%). Mp 267 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.10–8.15 (m, 2H, PhH), 7.43 (t, J = 8.6 Hz, 2H, PhH), 7.33 (d, J = 8.6 Hz, 2H, PhH), 6.97 (d, J = 8.6 Hz, 2H, PhH), 6.34 (s, 2H, NH_2), 4.92 (s, 2H, OCH_2), 3.92 (br s, 2H, NCH_2), 3.87 (br s, 2H, NCH_2), 3.65 (br s, 4H, $CON(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.4, 169.4, 165.9, 164.2 (d, J = 248.7 Hz), 162.2, 158.5, 156.9, 129.2 (d, J = 9.0 Hz), 129.1, 128.9 (d, J = 3.0 Hz), 116.5 (d, J = 21.6 Hz), 116.4, 97.6, 65.9, 44.9, 44.7, 43.5, 40.9 ppm. HRMS: calcd for $C_{23}H_{21}ClFN_6O_2S$ $[M+H]^+$ 499.11193, found 499.11023.

5.2.28. 7-*N*-Morpholinothiazolo[4,5-*d*]pyrimidin-5-amine (8)

This compound was synthesized from **4a** according to the procedure for the preparation of compound **11.** The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a white solid (70%). ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 9.45 (s, 1H, H-2), 6.29 (s, 2H, NH_2), 3.73–3.78 (m, 4H, $O(CH_2)_2$), 3.70–3.72 (m, 4H, $N(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 172.6, 162.0, 159.7, 159.1, 96.4, 65.8, 45.5 ppm.

5.2.29. 2,7-Dichlorothiazolo[4,5-*d*]pyrimidin-5-amine (10)

To a solution of 5-amino-2-chlorothiazolo[4,5-*d*]pyrimidin-7(6*H*)-one **9**[28] (1.3 g, 6.42 mmol) in phosphorus oxychloride (32 ml) was added *N,N*-dimethylaniline (3.2 ml). The mixture was heated at reflux for 2 h. After cooling to room temperature, the solvents were removed under reduced pressure. The crude residue was diluted with dichloromethane and poured into ice-water. The mixture was extracted with dichloromethane, washed with $NaHCO_3$ and brine, and dried over Na_2SO_4. After removing the solvents under reduced pressure, the residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a light yellow solid (0.48 g, 33%). Mp >285 °C decomposed. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.46 (s, 2H, NH_2) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 168.8, 162.8, 162.5, 152.6, 113.9 ppm. HRMS: calcd for $C_5H_3Cl_2N_4S$ $[M+H]^+$ 220.94555/222.94260, found 220.9448/222.94199.

5.2.30. 7-Chloro-N^2-(2-methoxyethyl)thiazolo[4,5-*d*]pyrimidine-2,5-diamine (11a)

To a solution of 2,7-dichlorothiazolo[4,5-*d*]pyrimidin-5-amine **10** (0.10 g, 0.45 mmol) and triethylamine (69 μl, 0.50 mmol) in dioxane (3 ml) was added 2-methoxyethylamine (39 μl, 0.45 mmol). The reaction mixture was heated at 70 °C for 2 h. After cooling, the volatiles were removed under reduced pressure. The crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 40:1) affording the title compound as a white solid (0.11 g, 94%). Mp 236–237 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 9.09 (s, 1H, NH), 6.71 (s, 2H, NH_2), 3.52 (br s, 4H, CH_2), 3.29 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ =171.8, 170.8, 162.3, 149.4, 107.6, 69.8, 57.9, 43.8 ppm. HRMS: calcd for $C_8H_9ClN_4O_2S$ $[M+H]^+$ 260.01347, found 260.03656.

5.2.31. *tert*-Butyl-4-(5-amino-7-chlorothiazolo[4,5-*d*]pyrimidin-2-yl)piperazine-1-carboxylate (11b)

This compound was synthesized in a yield of 97% according to the procedure for the preparation of compound **3a**, using *tert*-butyl piperazine-1-carboxylate. Mp >350 °C decomp. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 6.81 (s, 2H, NH_2), 3.64 (br s, 4H, $N(CH_2)_2$), 3.49 (br s, 4H, $CON(CH_2)_2$), 1.43 (s, 9H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 172.0, 171.7, 162.5, 153.7, 149.8, 107.8, 79.4, 47.7, 42.2, 28.0 ppm. HRMS: calcd for $C_{14}H_{20}ClN_6O_2S$ $[M+H]^+$ 371.10570, found 371.10496.

5.2.32. 2-(4-Fluorophenyl)-N^7-(2-methoxyethyl)thiazolo[4,5-*d*]pyrimidine-5,7-diamine (12a)

This compound was synthesized from **11a** according to the procedure for the preparation of compound **5a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a pale yellow solid (97%). Mp 225 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.95 (s, 1H, NH), 7.93–7.98 (m, 2H, ArH), 7.39 (t, J = 8.7 Hz, 2H, ArH), 6.37 (s, 2H, NH_2), 3.55 (br s, 4H, CH_2), 3.29 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): δ = 172.3, 171.3, 162.9 (d, J = 245.9 Hz), 162.8, 154.6, 134.3 (d, J = 3.0 Hz), 129.6 (d, J = 8.7 Hz), 115.7 (d, J = 21.6 Hz), 106.7, 69.9, 57.9, 43.8 ppm. HRMS: calcd for $C_{14}H_{13}FN_4O_2S$ $[M+H]^+$ 320.07432, found 320.09738.

5.2.33. *tert*-Butyl-4-(5-amino-7-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-2-yl)piperazine-1-carboxylate (12b)

This compound was synthesized from **11b** according to the procedure for the preparation of compound **5a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a pale yellow solid (94%). Mp 211 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 7.94–7.99 (m, 2H, ArH), 7.18 (t, J = 8.6 Hz, 2H, ArH), 5.08 (s, 2H, NH_2), 3.73 (br s, 4H, $N(CH_2)_2$), 3.59 (br s, 4H, $CON(CH_2)_2$), 1.49 (s, 9H, CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 173.4, 172.5, 164.1 (d, J = 294.5 Hz), 162.6, 156.8, 154.5, 134.0 (d, J = 3.1 Hz), 130.0 (d, J = 8.5 Hz), 116.0 (d, J = 15.9 Hz), 109.3, 80.9, 48.3, 43.1, 28.5 ppm. HRMS: calcd for $C_{20}H_{24}FN_6O_2S$ $[M+H]^+$ 431.16655, found 431.16558.

5.2.34. 7-(4-Fluorophenyl)-2-(piperazin-1-yl)thiazolo[4,5-*d*]pyrimidin-5-amine (13)

To a suspension of *tert*-butyl 4-(5-amino-7-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-2-yl)piperazine-1-carboxylate **12b** (0.30 g, 0.07 mmol) in dichloromethane (5 ml) was added dropwise trifluoroacetic acid (~5 ml) until the solid was completely dissolved. The reaction mixture was stirred at room temperature under a nitrogen atmosphere overnight. The solvents were evaporated in vacuo. The residue was resuspended in water and the solid was collected by filtration. The solid was washed with water, dried under vacuum, yielding the title compound as a white solid (0.175 g, 76%). Mp 280 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 7.96–8.01 (m, 2H, PhH), 7.39 (t, J = 8.7 Hz, 2H, PhH), 6.43 (s, 2H, NH_2), 3.58 (br s, 4H, $N(CH_2)_2$), 3.18 (s, 1H, NH), 2.81 (br s, 4H, $HN(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.3, 163.0 (d, J = 246.4 Hz), 162.9, 155.0, 134.1 (d, J = 3.0 Hz), 129.7 (d, J = 8.7 Hz), 115.8 (d, J = 21.5 Hz), 106.7, 49.4, 45.0 ppm. HRMS: calcd for $C_{15}H_{16}FN_6S$ $[M+H]^+$ 331.11412, found 331.11309.

5.2.35. 4-(5-Amino-7-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-2-yl)-*N*-m-tolylpiperazine-1-carboxamide (14a)

This compound was synthesized from **13** according to the procedure for the preparation of compound **7a**. The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a white solid (95%). Mp 228 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 8.62 (s, 1H, NH), 7.98–8.03 (m, 2H, PhH), 7.41 (t, J = 8.8 Hz, 2H, PhH), 7.31 (s, 1H, PhH), 7.27 (d, J = 7.3 Hz, 1H, PhH), 7.13 (t, J = 7.3 Hz, 1H, PhH), 6.78 (d, J = 7.3 Hz, 1H, PhH), 6.48 (s, 2H, NH_2), 3.73 (br s, 4H, $N(CH_2)_2$), 3.65 (br s, 4H, $CON(CH_2)_2$), 2.26 (s, 3H, CH_3) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.4, 172.2, 163.1 (d, J = 246.6 Hz), 162.9, 155.2, 154.8, 140.2, 137.4, 134.0 (d, J = 2.9 Hz), 129.7 (d, J = 8.7 Hz), 128.2, 122.6, 120.2, 116.8, 115.8 (d, J = 21.6 Hz), 106.8, 47.7, 43.1, 21.2 ppm. HRMS: calcd for $C_{23}H_{23}FN_7OS$ $[M+H]^+$ 464.16688, found 464.16510.

5.2.36. 1-(4-(5-Amino-7-(4-fluorophenyl)thiazolo[4,5-*d*]pyrimidin-2-yl)piperazin-1-yl)-2-(4-fluorophenoxy)ethanone (14b)

This compound was synthesized from **13**, according to the procedure for the preparation of compound **7b.** The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 50:1) to yield the title compound as a white solid (53 mg, 88%). Mp 266 °C. ^{1}H NMR (300 MHz, DMSO, 25 °C): δ = 7.97–8.02 (m, 2H, PhH), 7.41 (t, J = 8.8 Hz, 2H, PhH), 7.33 (d, J = 8.8 Hz, 2H, PhH), 6.98 (d, J = 8.8 Hz, 2H, PhH), 6.49 (s, 2H, NH_2), 4.93 (s, 2H, NCH_2), 3.78 (br s, 4H, NCH_2), 3.66 (br s, 4H, $CON(CH_2)_2$) ppm. ^{13}C NMR (75 MHz, DMSO, 25 °C): 172.4, 172.1, 165.9, 163.1 (d, J = 246.7 Hz), 163.0, 156.9, 155.3, 134.0 (d, J = 2.9 Hz), 129.7 (d, J = 8.7 Hz), 129.1, 124.5, 116.4, 115.8 (d, J = 21.6 Hz), 106.8, 65.9, 47.6, 43.3 ppm. HRMS: calcd for $C_{23}H_{21}ClFN_6O_2S$ $[M+H]^+$ 499.11193, found 499.11023.

5.2.37. *tert*-Butyl 4-(5-acetamidothiazolo[4,5-*d*]pyrimidin-2-yl)piperazine-1-carboxylate (15)

A solution of *tert*-butyl 4-(5-amino-7-chlorothiazolo[4,5-*d*]pyrimidin-2-yl)piperazine-1-carboxylate **11b** (40 mg, 0.11 mmol) and 10% Pd/C (3 mg) in THF/MeOH (2:1, 1 ml) was stirred at room temperature under H_2 for 4 h. The mixture is filtered over Celite and solvent are evaporated in vacuo. The crude product was dissolved in acetic anhydride and heated at 80 °C for 3 h. After removing the solvent, the crude residue was purified by chromatography on silica gel (CH_2Cl_2/MeOH 50:1) to yield the title compound as a white solid (13 mg, 32%). ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 8.67 (s, 1H, H-7), 8.62 (s, 1H), 3.76 (br s, 4H, $N(CH_2)_2$), 3.61 (br s, 4H, $CON(CH_2)_2$), 2.55 (s, 3H, $COCH_3$), 1.49 (s, 9H, $O(CH_3)_3$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 173.8, 170.5, 156.1, 154.3, 148.5, 117.6, 80.8, 48.4, 43.0, 28.3, 25.2 ppm.

5.2.38. 7-Ethoxy-N^2-(2-methoxyethyl)thiazolo[4,5-*d*]pyrimidine-2,5-diamine (16)

5.2.38.1. Method A. This compound was synthesized from **11a** according to the procedure for the preparation of compound **1b.** The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a white solid (70%).

5.2.38.2. Method B. This compound was synthesized from **4b** according to the procedure for the preparation of compound **11a.** The crude residue was purified by flash chromatography on silica (CH_2Cl_2/MeOH 40:1) to yield the title compound as a white solid (82%). Mp 205 °C. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C): δ = 6.32 (s, 1H, NH), 4.86 (s, 2H, NH_2), 4.43 (q, J = 7.1, 2H, CH_2CH_3), 3.61–3.67 (m, 4H, CH_2CH_2), 3.38 (s, 3H, OCH_3), 1.38 (t, J = 7.1 Hz, 3H, OCH_2CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C): δ = 172.4, 172.2, 163.9, 161.9, 95.1, 70.6, 62.5, 59.0, 44.9, 14.8 ppm. HRMS: calcd for $C_{10}H_{16}N_5O_2S$ $[M+H]^+$ 270.10247, found 270.10168.

5.3. Protein overexpression and purification

5.3.1. *E. coli* and *S. aureus* SecA

E. coli and *S. aureus* SecA were overexpressed as histidine-tagged fusion proteins in *E. coli* BL21.19 cells. Cell pellets were suspended in buffer A (50 mM Tris–HCl, 1 M NaCl, 10% glycerol, 5 mM imidazole, pH 8.0) containing 2.5 mM PMSF and 50 μg/ml DNase. After lysis by passing the cells three times through a French pressure cell (69 MPa), cell debris was removed by centrifugation. The cleared extract was applied to a nickel-nitrilotriacetic acid (Ni-NTA) column pre-equilibrated with buffer A. After washing the column with 10 column volumes buffer A and 5 column volumes buffer B (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 5 mM imidazole, pH 8.0), proteins were eluted by applying 10 column volumes of buffer C (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 100 mM imidazole, pH 8.0). Peak fractions containing recombinant SecA were pooled and dialysed against buffer D (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 2 mM EDTA, pH 8.0). Protein samples were concentrated using Vivaspin centrifugal devices (Sartorius, Göttingen, Germany) with a MWCO of 3 kDa and dialysed against buffer E (50 mM Tris–HCl, 50 mM NaCl, 50% glycerol, 2 mM EDTA, pH 8.0). Enzyme preparations were aliquoted and stored at −80 °C until use.

5.3.2. *E. coli* preprotein AlkProPhoA(Cys-)

The preprotein AlkProPhoA(Cys-) from *E. coli* was overexpressed in *E. coli* BL21.19 and purified as described.[39,40] Briefly, cell pellets were suspended in buffer F (50 mM Tris–HCl, 0.5 M NaCl, 5% glycerol, pH 8.0) containing 2.5 mM PMSF and 50 μg/ml DNase. After lysis by passing the cells three times through a French pressure cell (69 MPa), ProPhoA(Cys-) was isolated as inclusion bodies and purified by Ni-NTA affinity chromatography under denaturing conditions. Peak fractions containing the recombinant preprotein (in 50 mM Tris–HCl, 50 mM NaCl, 5% glycerol, 100 mM imidazole, 6 M urea, pH 8.0) were pooled and dialysed against 50 mM Tris–HCl, 50 mM KCl, 5% glycerol, 6 M urea, 1 mM EDTA, pH 8.0. Enzyme preparations were aliquoted and stored at −80 °C until use.

5.3.3. Isolation of inner membrane vesicles

Inner membrane vesicles (IMVs) were prepared from *E. coli* strain BL31(DE3) using pET610 to overexpress SecYEG.[41] Cells pellets were suspended in 30 ml 50 mM Tris–HCl, 20% glycerol, pH 8.0 containing 2.5 mM PMSF and 50 μg/ml DNase (buffer F). Cells were lysed by three passages through a French pressure cell (69 MPa), diluted with an equal volume of buffer F and centrifuged (4000*g*, 10 min, 4 °C) to remove cell debris. Membranes were collected from the supernatant by ultracentrifugation (35,000*g*, 90 min, 4 °C). The resulting pellet was resuspended in buffer F and loaded onto a five-step sucrose gradient consisting of 1.1, 1.3, 1.5, 1.7 and 1.9 M sucrose layers (in 50 mM Tris–HCl, pH 8.0). After ultracentrifugation (24,000*g*, 16 h, 4 °C), the brown fraction containing the IMVs was collected, diluted in 300 ml buffer F and recollected by ultracentrifugation (35,000*g*, 90 min, 4 °C). Purified IMVs were suspended in 5 ml 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 8.0 (buffer G). To inactivate endogenous SecA, IMVs were treated with 6 M urea (30 min, 4 °C). Urea-stripped IMVs were sedimented (35,000*g*, 35 min, 4 °C) through a sucrose cushion (0.2 M sucrose, 50 mM Tris–HCl pH 8.0, 50 mM KCl), washed with buffer G, and after a second centrifugation step resuspended into 5 ml buffer G. The suspension was homogenized with a homogenizer and the membrane suspension was passed through a lipid extruder for better homogenization. Protein concentrations were estimated by the Bradford assay, using bovine serum albumin as the standard. SecA-stripped IMVs were stored in small aliquots at −80 °C.

5.4. SecA ATPase activity measurements

The ATPase activity of SecA was quantified in 96-well microtiter (50 μl reaction volume) plates using the malachite green colorimetric method for the detection of free inorganic phosphate (P_i).[38] *E. coli* SecA intrinsic ATPase activity measurements were performed at 37 °C in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 0.5 μM (50 μg/ml) ecSecA, 100 μM ATP, pH 8.0. *S. aureus* SecA intrinsic ATPase activity measurements were performed at 28 °C in 50 mM HEPES, 50 mM KCl, 2 mM $MgCl_2$, 0.4 mg/ml BSA, 4 μM (400 μg/ml) saSecA, 100 μM ATP, pH 7.5. *E. coli* SecA translocation ATPase activity measurements were performed at 37 °C in 50 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.4 mg/ml BSA, 0.2 μM (20 μg/ml) ecSecA, *E. coli* IMVs containing recombinant ecSecYEG (12 μg/ml protein), *E. coli* ProPhoA(Cys-) (40 μg/ml), 100 μM ATP, pH 8.0.

Initial rates of ATP hydrolysis were measured in the presence of 200 μM compound or DMSO (2% final DMSO concentration). For background correction, all reactions were performed with negative controls with no SecA in the reaction mixture. After incubation (2 h for intrinsic ATPase, 30 min for translocation ATPase activity measurements) at the appropriate temperature, ATPase reactions were stopped by adding 150 μl Malachite Green Reagent (Enzo LifeSciences, Plymouth, USA) to the reaction mixture. Microtiter plates were incubated for 20 min at room temperature and read at 660 nm using a Tecan Infinite M200 microplate reader. After background correction, rates of SecA ATP hydrolysis in the presence of compound were expressed relative to rate of ATP hydrolysis in the absence of compound.

For the determination of the K_i value of compound **14b** for inhibition of the ecSecA translocation ATPase activity, initial rates of ATP hydrolysis were measured as described above, in the presence of different inhibitor (25, 50, 100 and 200 μM) and ATP concentrations (0–2 mM). Apparent K_m and V_{max} values were estimated by fitting the data by nonlinear regression analysis to the Michaelis–Menten equation. The K_i value of compound **14b** for inhibition of the ecSecA translocation ATPase activity was determined by linear regression using Dixon plot analysis ($1/v$ vs [I]).

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