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Vectorization Efforts To Increase Gram-Negative Intracellular Drug Concentration: A Case Study on HldE-K Inhibitors

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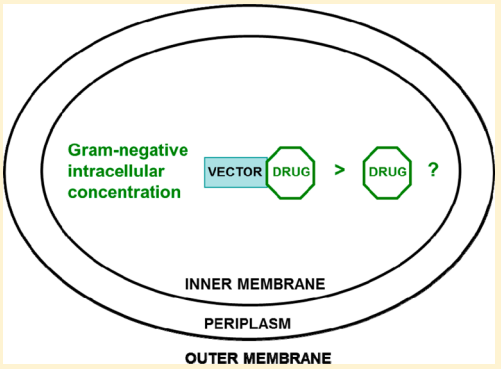
ABSTRACT: In this paper, we present different strategies to vectorize HldE kinase inhibitors with the goal to improve their Gram-negative intracellular concentration. Syntheses and biological effects of siderophoric, aminoglycosidic, amphoteric, and polycationic vectors are discussed. While siderophoric and amphoteric vectorization efforts proved to be disappointing in this series, aminoglycosidic and polycationic vectors were able for the first time to achieve synergistic effects of our inhibitors with erythromycin. Although these effects proved to be nonspecific, this study provides information about the required stereoelectronic arrangement of the polycationic amines and their basicity requirements to fulfill outer membrane destabilization resulting in better erythromycin synergies.



INTRODUCTION

Rates of multidrug-resistant Gram-negative organisms are increasing at an alarming speed, outpacing in some cases those of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE).[1] This, combined with the shortage of new drugs in the antibacterial development pipeline,[2] constitutes a major health concern.

One approach to address this issue is to identify inhibitors with novel modes of action based on the new targets originating from the rise of bacterial genomics in the 1990s.[3] Unfortunately, the general outcome has proven disappointing so far,[4] some authors even cautioning against the exploitation of cytosolic targets with regard to the difficulty of reaching the required active concentration in the bacterial cell.[5] Indeed in Gram-negative bacteria, obtaining the active intracellular drug concentration with reversible synthetic inhibitors represents a formidable challenge, since the compound must first cross a hydrophilic outer membrane (OM)[6] followed by a lipophilic inner one (IM) and finally resist a battery of efflux mechanisms.[7−9]

We have previously reported[10] the nanomolar optimization of 1,2,4-triazine-based inhibitors of HldE-kinase, a novel antivirulence target involved in the synthesis of the inner core of lipopolysaccharides (LPS).[11] Some of these compounds demonstrate specific LPS biosynthetic inhibition on efflux-deleted Gram-negative strains, yet are inactive at relevant pharmacological doses on wild pathogens because of poor membrane permeation and/or efficient efflux. A potential method to circumvent these problems is the adjunction of functional groups or covalently attached molecules called vectors to the inhibitor in the hope of increasing intracellular drug concentration by improving membrane permeation and/or reducing efflux (Figure 1).

Several vector-mediated drug delivery systems are known and may be classified depending on their mode of uptake in bacteria. Among them, siderophoric vectorization aims at attaching a siderophore-like functionality to the drug to take advantage of the bacterial iron-uptake mechanism, a strategy sometimes referred to as the "Trojan Horse" approach.[12−14] With a siderophoric moiety bound to their benzothiazolyl group, compounds **1**−**8** in Table 1 illustrate this strategy.

By tethering an aminoglycoside moiety to the drug, aminoglycosidic vectorization targets the OM passage via porins for the smaller-size aminoglycosides and/or by self-promoted pathway via displacement of the stabilizing dications of the LPS.[15] Then in a strategy reminiscent of the "Trojan Horse" approach, the IM would be crossed by an energy-dependent aminoglycoside uptake.[16] The aminoglycoside kanamycin was attached to the benzothiazole part of our series via a triazole linker to illustrate this strategy (compound **9**, Table 2).

Amphoteric antibacterials such as some fluoroquinolones or penicillins penetrate the OM via passive diffusion[17] through the porins in their zwitterionic forms,[18,19] a process facilitated in some cases by formation of a Mg^{2+} chelate.[20] Passage though the IM for amphoteric fluoroquinolones or tetracyclines is subsequently achieved by diffusion of the equilibrated neutral microspecies.[20,21] Therefore, amphoteric vectorization, consisting of installing acidic and/or basic functional groups to an inhibitor to make it amphoteric, could lead to better OM penetration via its zwitterionic form and better IM influx via its uncharged neutral form. To illustrate this strategy, compounds **10** and **11** in Table 3 were designed with a basic amine and an

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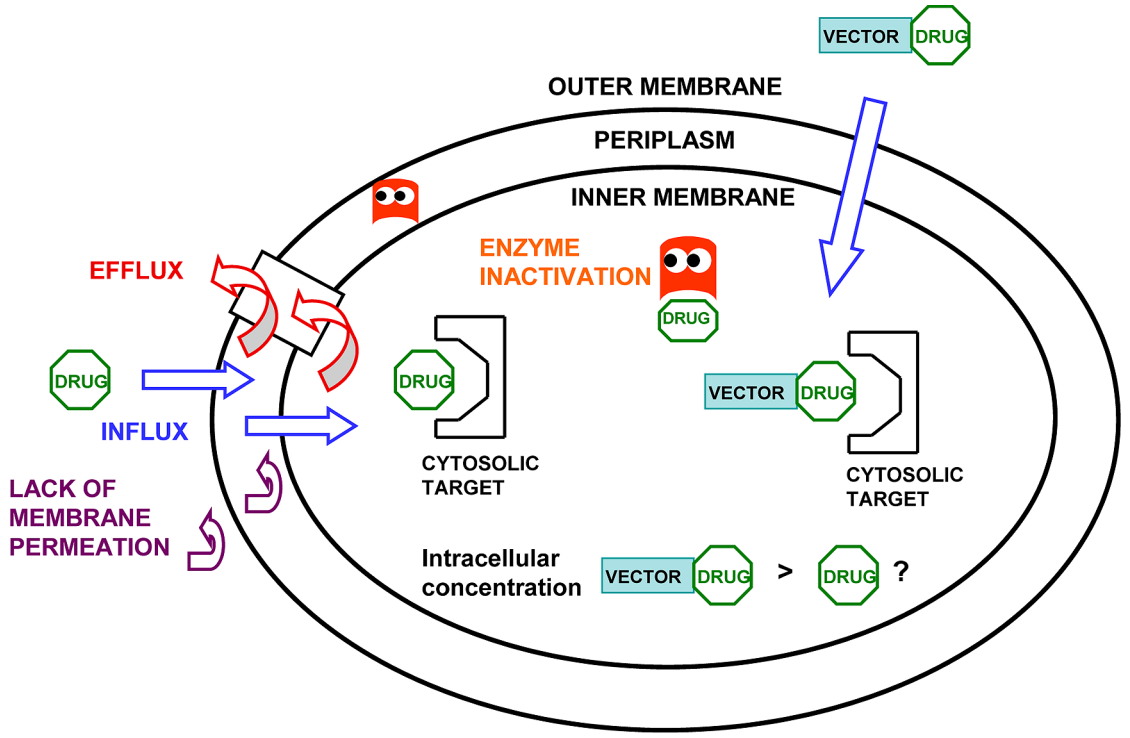

Figure 1. Schematic representation of the mechanisms affecting Gram-negative drug intracellular concentration.

acidic phenolic group to allow them to exist partly in both zwitterionic and neutral forms.

The self-promoted uptake involves the displacement of LPS-cross-bridging cations by polycationic compounds, allowing their entry into the periplasm after LPS destabilization.[15,22,23] Polycationic vectorization is the process of adding cationic functional groups to a drug in order to increase its OM permeation. In some instances, such chemical modifications have been shown to broaden the in vitro activity spectrum of glycopeptides against Gram-negative pathogens.[24,25] To illustrate this strategy, compounds **12**–**18** in Table 4 have been appended with polyamine side chains.

Encouraged by SAR data[10] and crystallographic results[26] indicating space available at both extremities of our HldE-K inhibitors, we embarked on a vectorization program using these strategies. In the present report, we describe the syntheses, biological results, and the effects of these vectors on Gram-negative intracellular concentration of our inhibitors.

CHEMISTRY

The vectorization of our reported HldE-K inhibitors[10] was carried out using concise and straightforward synthetic routes. Acidic compound **19**[10] was derivatized using standard coupling reagents in amides or esters as illustrated in Scheme 1. Reaction with hydroxylamine provided the hydroxamic acid derivative **1**. Esterification with alcohol **20**[27] led to ester **21**, which was deprotected under acidic conditions to afford compound **6**, while amidification with 1,4-bis-Boc-1,4,7-triazaheptane led to amide **22**, which was deprotected under acidic conditions to afford compound **14**. Similar conditions were used to synthesize target amides **15**, **16**, and **18**.

Compound **2** was accessed by nucleophilic substitution of chlorotriazine **23**[10] by aminomethylthiazole **24**[10] followed by Boc and THP deprotection and final carbamoylation as illustrated in Scheme 2.

Sulfonylation of amino compound **27**[10] provided derivative **28** that was demethylated to afford compound **3**, whereas its amidation using standard coupling reagents provided compound **8** as illustrated in Scheme 3. Compound **5** was obtained in a similar way by coupling **27** with comenic acid.

Hydroxy compound **29**[10] was derivatized as illustrated in Scheme 4 by mesylation followed by substitution using a thionucleophile to provide compound **7** or using amino-nucleophile **32**[28] to afford after Boc deprotection compound **17**. The low reported yield of this deprotection might be explained by purification over a strong cation exchange column (SCX), which may have partially retained the product. Derivative **13** was obtained in a similar way using 1-methylpiperazine as nucleophile.

Propargylamide derivative **34**, prepared from acid **19**[10] using similar conditions as described in Scheme 1, was coupled to N-protected 6″-azido-kanamycin A **33**[29] under copper-catalyzed Huisgen conditions to afford compound **35**. This cycloaddition was run according to the mentioned reference, which did not use any nitrogen-type ligands to accelerate the reaction. Since intermediate **35** was prepared in a sufficient amount for the final deprotection and biological evaluation, the reported yield of 12% was not further optimized. Final Boc removal under acidic conditions provided compound **9** as illustrated in Scheme 5.

Phenol derivative **10** was obtained by demethylation of the corresponding methyl ether analogue **36**[10] using Lewis acidic conditions as illustrated in Scheme 6. Compound **11** was prepared following the same procedure from the amido analogue **37**.[10]

Aminoethyl derivative **12** was prepared via nucleophilic substitution of chlorotriazine **38**[10] by amine **24**[10] followed by Boc removal, primary amine protection, aniline substitution with 2-chloro-*N*,*N*-dimethylethylamine, and final deprotection as illustrated in Scheme 7. The 13% reported yield for the final

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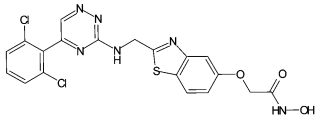
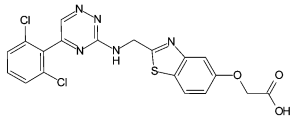
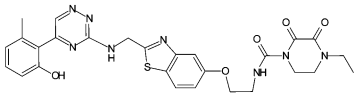
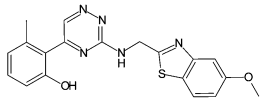
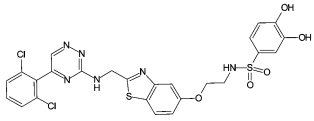
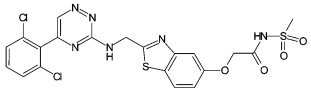
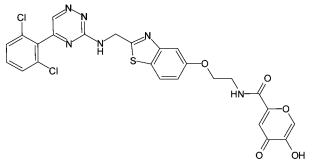
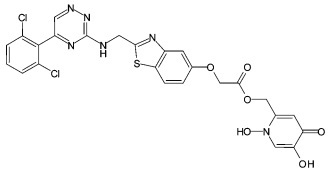
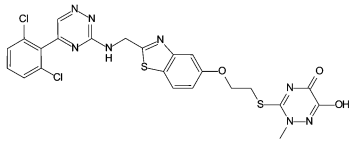
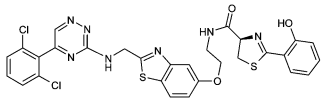
Table 1. Siderophoric Vectorization: Comparison of Vectorized and Unvectorized Compounds

Compd	Structure	IC_{50} (nM)	Synergy with Erythromycin MIC (μg/mL)		
		E. coli HldE-K	*E. coli* K12 Δ-*acrAB*, Δ-*tolC* [a]	*E. coli* K12 Δ-*acrAB*, Δ-*tolC* Iron depleted [b]	*E. coli* K1 wild [c]
1		11	2	1	>64
19		19	32	N/A	>64
2		520	>32	8	>32
39[10]		69	8	N/A	>32
3		33	4	2	>64
4[10]		16	16	16	>64
5		71	>64	2	>64
6		30	>64	32	>64
7		25	2	1	>64
8		660	>32	>32	>32

[a]Synergistic MIC in the presence of 1 μg/mL erythromycin. [b]Synergistic MIC in the presence of 0.1 μg/mL erythromycin in iron depleted medium. N/A: not applicable. [c]Synergistic MIC in the presence of 8 μg/mL erythromycin.

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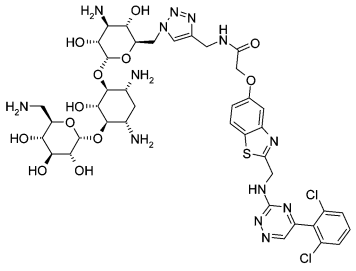
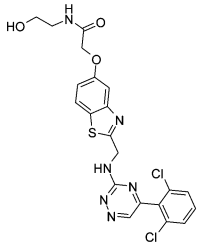
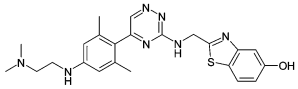
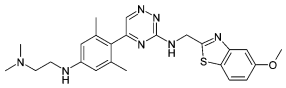
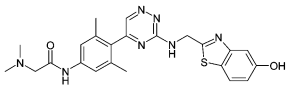
Table 2. Aminoglycosidic Vectorization: Comparison of Vectorized and Unvectorized Compounds

Compd	Structure	IC_{50} (nM)	Synergy with Erythromycin MIC (μg/mL)		
		E. coli HldE-K	*E. coli* K12 Δ-*acrAB*, Δ-*tolC* -[a]	*E. coli* K1 Δ-*tolC*[a]	*E. coli* K1 wild[b]
9		98	4	2	8
40[10]		55	2	16	>64

[a]Synergistic MIC in the presence of 1 μg/mL erythromycin. [b]Synergistic MIC in the presence of 8 μg/mL erythromycin.

Table 3. Amphoteric Vectorization: Comparison of Vectorized and Unvectorized Compounds

Compd	Structure	IC_{50} (nM)	Synergy with Erythromycin MIC (μg/mL)		Species distribution pH 7.4[c] (%)	
		E. coli HldE-K	*E. coli* K12 Δ-*acrAB*, Δ-*tolC*[a]	*E. coli* K1 wild[b]	Z[d]	N[e]
10		780	16	>64	13.3	1.5
36		82	32	>64	N/A[f]	1.8
11		880	16	>64	6.6	44.3

[a]Synergistic MIC in the presence of 1 μg/mL erythromycin. [b]Synergistic MIC in the presence of 8 μg/mL erythromycin. [c]Species distribution based on predicted pK_a microconstants (ACDLabs, version 12). [d]Zwitterionic form. [e]Neutral form (uncharged). [f]Not applicable.

one-pot two steps of this procedure might be explained by the low nucleophilic reactivity of the aniline-type nitrogen in the first-step nucleophilic substitution, which could be further deactivated by the electron-deficient triazine.

RESULTS AND DISCUSSION

The biological activities of the compounds were determined by measuring their enzymatic inhibitory concentrations on *E. coli* HldE-K expressed as IC_{50}, as well as their ability to synergize the Gram-negative antibacterial potential of erythromycin expressed as a synergistic MIC of inhibitor in association with erythromycin at its own MIC/4 concentration against *E. coli* K12 MG1655 Δ-*acrAB*-Δ*tolc*, *E. coli* K1 (018:K1:H7) wild type, and Δ*tolc*.[10,30] In both mutant strains, deletion of the major efflux pump AcrAB/TolC (in part or entirely) leads to an increased susceptibility toward amphiphilic and lipophilic antibiotics.[31] In the most promising synergy cases, bacterial LPS electrophoresis experiments[10] were carried out with the inhibitor alone to determine its capacity to specifically inhibit

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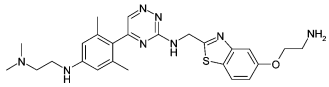
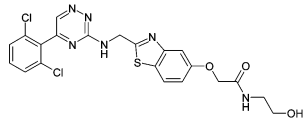
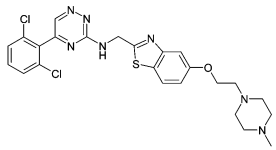
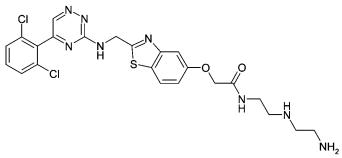
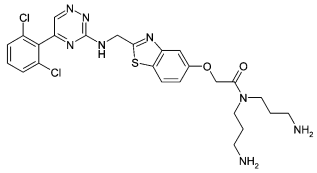
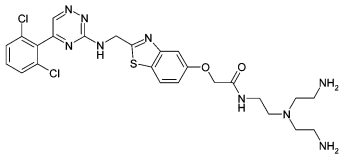
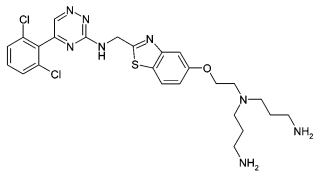
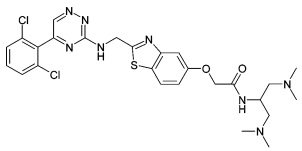
Table 4. Polycationic Vectorization: Comparison of Vectorized and Unvectorized Compounds

Compd	Structure	IC_{50} (nM)	Synergy with Erythromycin MIC (μg/mL)		Species distribution pH 7.4[c] (%)		
		E. coli HldE-K	*E. coli* K1 Δ-*tolC*[a]	*E. coli* K1 wild[b]	N[d]	M[e]	D[f]
12		1400	>64	>64	0	15	85
41[10]		55	16	>64	100	0	0
13		110	32	>64	37	63	0
14		110	8	32	0	96	4
15		510	4	8	0	1	99
16		580	4	8	0	3	97
17		630	2	4	0	0	98[g]
18		420	32	>64	6	86	8

[a]Synergistic MIC in the presence of 1 μg/mL erythromycin. [b]Synergistic MIC in the presence of 8 μg/mL erythromycin. [c]Species distribution based on predicted pK_a microconstants (ACDLabs, version 12). [d]Neutral form (uncharged). [e]Monocationic form. [f]Dicationic form. [g]Along with 2% of the tricationic form.

full LPS biosynthesis and therefore provide a marker for its intracellular concentration.

Since hydroxamate-type siderophores are known to be recognized by *E. coli* FhuD binding protein,[32] the carboxylic acid moiety of **19** was transformed into an iron-chelating hydroxamic acid group in compound **1** with the hope of benefiting from this active transport system (Table 1). While enzymatic potency was preserved, a 16-fold gain in synergy with erythromycin was achieved on *E. coli* K12 Δ-*acrAB*, Δ-*tolC*, an efflux-deleted strain. Repeating the synergy experiment under iron-depleted conditions to maximize the expression of bacterial siderophoric influx did not lead to any significant

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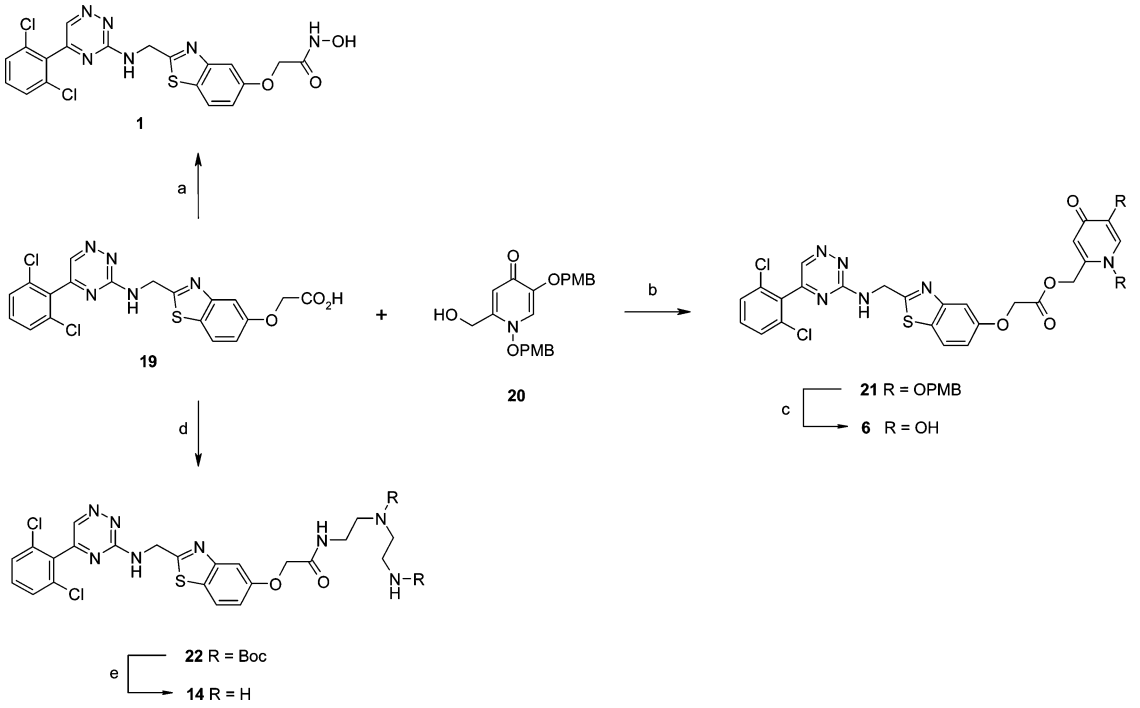
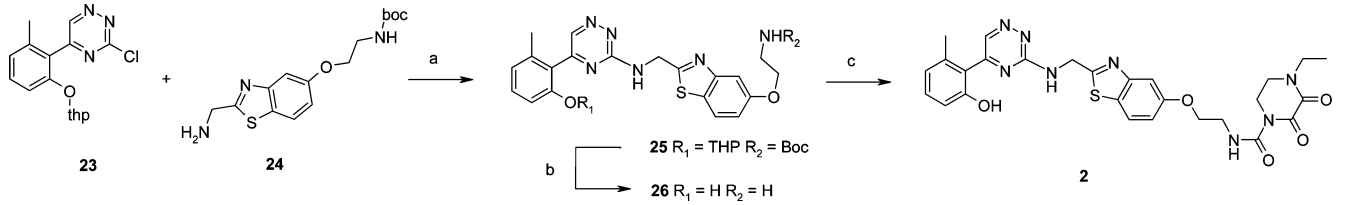
Scheme 1. Derivatization of Compound 19[a]



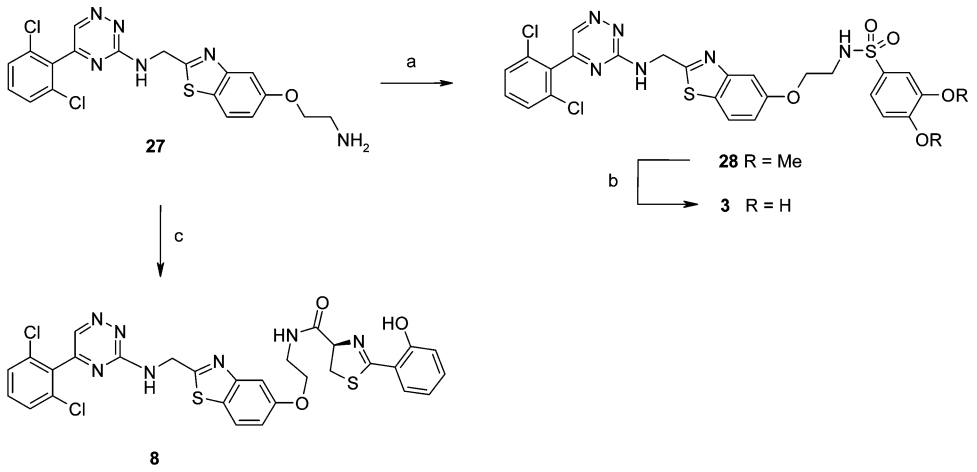
[a]Reagents: (a) hydroxylamine, DIPEA, EDCI, HOBt, DMF, rt (37%); (b) EDCI, DMAP, THF, rt (32%); (c) TFA, DCM, rt (46%); (d) 1,4-bis-Boc-1,4,7-triazaheptane, EDCI, HOBt, DIPEA, DMF, rt (56%); (e) HCl, dioxane, methanol, DCM, rt (63%).

Scheme 2. Synthesis of Compound 2[a]



[a]Reagents: (a) DIPEA, ACN, 85 °C (45%); (b) HCl, dioxane, 0 °C to rt (62%); (c) 4-ethyl-2,3-dioxo-1-piperazinecarbonyl chloride, DIPEA, THF, 0 °C to rt (29%).

Scheme 3. Derivatization of Compound 27[a]



[a]Reagents: (a) 3,4-dimethoxybenzenesulfonyl chloride, DIPEA, DCM, 0 °C to rt (78%); (b) BBr_3, DCM, −78 to −30 °C (20%); (c) (*R*)-2-(2-hydroxyphenyl)-4,5-dihydro-1,3-thiazole-4-carboxylic acid, EDCI, DMAP, DIPEA, HOBt, rt (30%).

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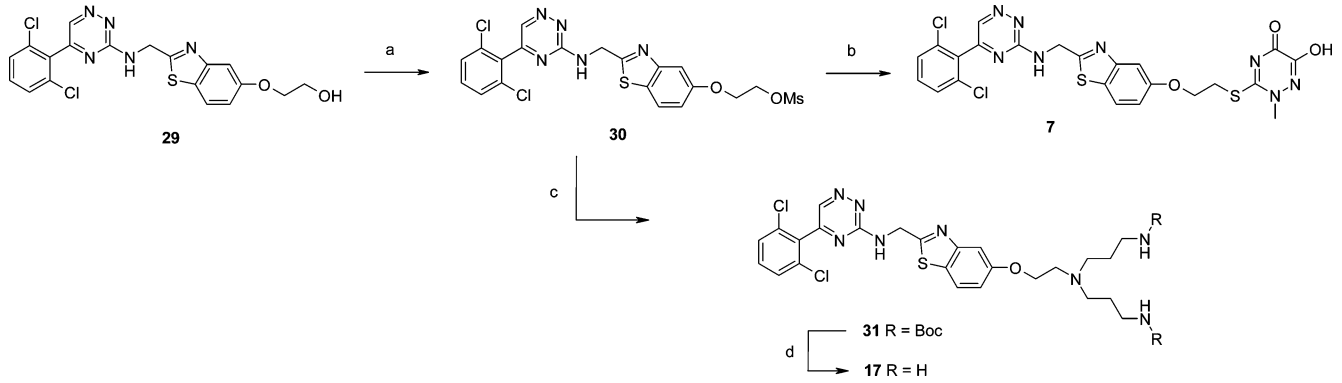
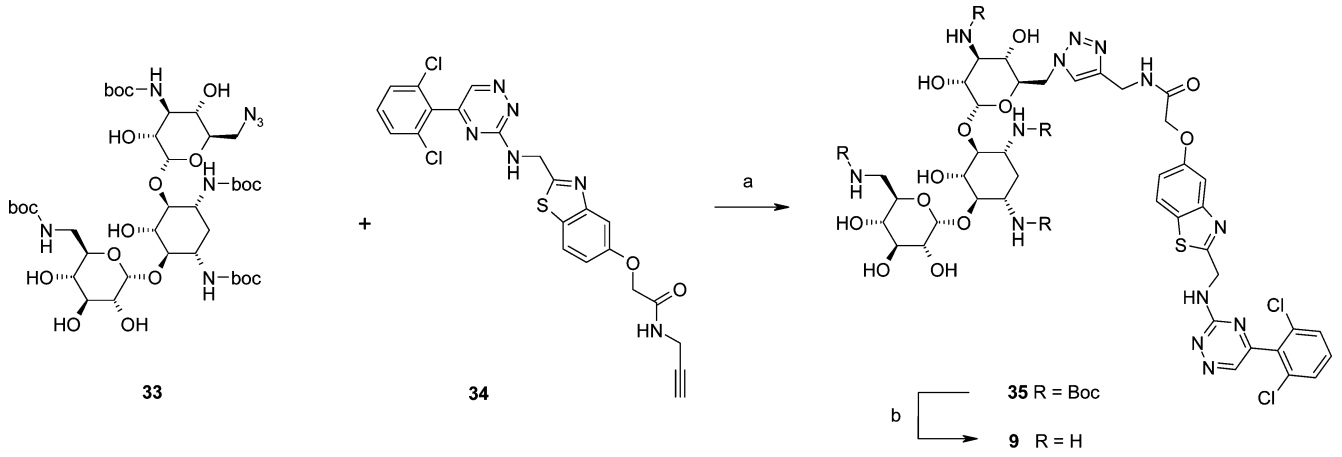
Scheme 4. Derivatization of Compound 29[a]



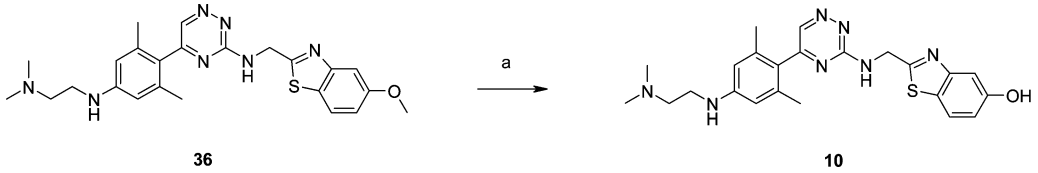
[a]Reagents: (a) MsCl, TEA, THF, 0 °C to rt (90%); (b) 6-hydroxy-3-mercapto-2-methyl-2*H*-[1,2,4]triazin-5-one, TEA, DMF, 60 °C (28%); (c) [3-(3-*tert*-butoxycarbonylaminopropylamino)propyl]carbamic acid *tert*-butyl ester **32**, ACN, 85 °C (43%); (d) HCl, dioxane, DCM, 0 °C to rt (23%).

Scheme 5. Synthesis of Kanamycin Derivative 9[a]



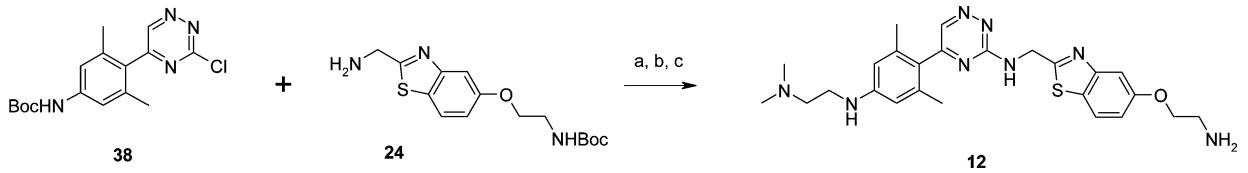
[a]Reagents: (a) $CuSO_4$, sodium ascorbate, *tert*-BuOH, water, rt (12%); (b) HCl, dioxane, MeOH, rt (64%).

Scheme 6. Synthesis of Compound 10[a]



[a]Reagents: (a) BBr_3, DCM, −78 °C (44%).

Scheme 7. Synthesis of Compound 12[a]



[a]Reagents: (a) DIPEA, ACN, 85 °C (58%); (b) (i) HCl (aqueous 37%), 0 °C; (ii) $(Boc)_2O$, TEA, THF, rt (73%); (c) (i) 2-chloro-*N*,*N*-dimethylethylamine hydrochloride, $NaHCO_3$, NaI, EtOH, 80 °C; (ii) HCl, dioxane, MeOH, rt (13%).

improvement. This cast some doubts of whether the improvement of erythromycin synergy seen with **1** vs **19** was truly due to siderophoric uptake followed by specific cytosolic inhibition of HldE-K. In this case, the observed synergy might possibly be explained by the hydroxamate-induced displacement of LPS-stabilizing cations. Adding other iron-chelating moieties to the right part of our unvectorized inhibitors such as catechol (**3**), acetylsulfamoyl (**4**), 1,5-dihydroxy-4-pyridone (**6**), and 6-hydroxy-2-methyl-2*H*-[1,2,4]triazin-5-one (**7**) led to similar results. Enzymatic activity could be maintained in the low

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nanomolar range, and in some instances potent synergistic activity was obtained on *E. coli* K12 Δ-*acrAB*, Δ-*tolC* (2 μg/mL for **7** vs 32 μg/mL for **19** with comparable enzymatic activities). Yet again, iron-depleted conditions could not significantly improve these synergies. Although some of these vectors have been able to achieve good periplasmic accumulation through demonstrated siderophoric influx,[33] they could not lead in our series to enhanced cytosolic concentrations under iron-depleted conditions nor on wild-type *E. coli* K1 strain. Tethering in compound **8** the 2-(2-hydroxy-phenyl)-4,5-dihydrothiazolyl moiety commonly found in Gram negative siderophores such as yersiniabactin or pyochelin led to a decrease in enzymatic activity and a complete loss of synergistic activity on our bacterial strains. Adding to compound **39** the piperazinedione vector found in piperacillin also led to a decrease in enzymatic activity (520 nM in **2** vs 69 nM in **39**). However, for this compound the synergistic bacterial activity on *E. coli* K12 Δ-*acrAB*, Δ-*tolC* could be significantly improved under iron-depleted conditions, indicating a possible true siderophoric uptake. A similar significant improvement of synergistic activity under iron-depleted conditions was observed for **5**, which is vectorized with a kojic acid derivative known for its iron-chelating properties.[34,35] Compound **5** also presents an interesting enzymatic potency below 100 nM. These two promising compounds unfortunately were found to be inactive on wild *E. coli* K1 strain, even under iron-depleted conditions. These results indicate that our vectors based on the siderophoric strategy, although displaying in some instances improvements on efflux-deleted strains, are not potent enough to enhance the cytosolic concentration of our compounds in wild strains. This may suggest the need for a more complete siderophoric moiety or some drug-release mechanism in the periplasm to better cross the IM.[36]

Aminoglycosidic vectorization was then attempted by tethering the aminoglycoside kanamycin to a representative member of our series. The resulting compound **9** (Table 2) still displayed potent enzymatic activity despite the added steric bulk. More interestingly, synergy with erythromycin was achieved for the first time on *E. coli* K1 wild strain (compare **9** and **40**). Control experiments were run to assess that the kanamycin moiety did not impart any antibacterial activity in **9** at these doses.[30] Control checkerboard assays were also run to rule out any potential synergies between the unvectorized compound **41** and erythromycin or between the vector itself, kanamycin, and erythromycin.[37] On *E. coli* K1 wild strain, the MIC of erythromycin remained at 16 μg/mL independent of the concentration of **41** (0−32 μg/mL). On the same strain, kanamycin alone displayed a MIC of 8 μg/mL, while it demonstrated a pure additive effect in combination with erythromycin. In view of this promising result, an LPS electrophoresis assay was then run to determine the ability of **9** to inhibit LPS biosynthesis on this strain. However, at 30 μg/mL, no inhibition of LPS biosynthesis was observed, suggesting that the synergy with erythromycin is not specific. One could speculate that this effect is probably induced by the amphiphilic character of **9**, which may facilitate the permeation of erythromycin.

Enterobacterial porins such as OmpF display a hydrophilic channel with negative residues facing the opposite half-ring of positively charged ones.[38,39] This channel constitutes a stereoelectronic filter selecting small polar molecules, favoring in some instances the translocation of zwitterionic compounds.[40] To increase the polarity of our inhibitors and enhance their charge distribution, an acidic phenolic hydroxyl was positioned to the right part of **36**, while the basic amine left part was conserved. The resulting compound (**10**, Table 3) presents 13.3% of zwitterionic form at the bacterial assay pH. Despite a 10-fold enzymatic potency loss, **10** displayed a slightly better synergistic activity with erythromycin on *E. coli* K12 Δ-*acrAB*, Δ-*tolC* in comparison to the nonzwitterionic analogue **36**. However, this compound proved to be inactive on *E. coli* K1 wild strain. Concerned that the low amount of uncharged neutral form of **10** (1.5% at pH 7.4, Table 3) might be a limitation to cross the IM, compound **11** was designed with a higher percentage of neutral form (44.3%) while still harboring a significant amount of zwitterionic form (6.6%). Here again despite a 10-fold enzymatic potency loss, **11** displayed a slightly better synergistic activity with erythromycin on *E. coli* K12 Δ-*acrAB*, Δ-*tolC* in comparison to the nonzwitterionic analogue **36** but was still inactive on the wild strain. The results of amphoteric vectorization therefore proved to be disappointing in our series because of the enzymatic activity loss imparted by this strategy, preventing it from expressing its real potential. Another factor may also be the inadequate geometry of our charge-bearing groups, since stereoelectronic complementarity between the compound and the channel should be present for proper translocation.[41]

Adjunction of cationic groups to our representative inhibitors was then attempted with the hope of penetrating the bacterial cell via membrane destabilization. Initial trials with protonable amines at both sides of our compounds resulted in a marked decrease of enzymatic activity and an absence of synergy with erythromycin (compound **12**, Table 4). The presence of 85% of the dicationic form of **12** was not able to permeabilize the antibiotic in the bacteria, suggesting that another stereoelectronic arrangement of the charges, possibly closer to each other or in a more "surfactant-like" arrangement, is required to achieve membrane destabilization. To reach this goal, the charges were positioned to the right side of our inhibitors. In compound **13**, the piperazine nitrogens mutually decrease their pK_a by inductive effect and no dicationic form is predicted at pH 7.4. Despite displaying potent enzymatic activity, this amine disposition did not enhance the synergy with erythromycin in comparison to the unvectorized analogue **41**. Increasing the basicity of the protonable amines (**14**, **15**) and their numbers (**16**, **17**) resulted in an increase of the dicationic form, culminating with the presence of a small amount of the tricationic form in **17**. This was directly correlated with a marked improvement of the synergistic activity with erythromycin, even on the wild strain. These data also indicate that some flexibility in the amine disposition is tolerated as long as a high level of the dicationic form is present. Here again, a control checkerboard assay was run to rule out any potential synergies between the polycationic vector of compound **17** and erythromycin.[42] On *E. coli* K1 wild strain, the MIC of erythromycin remained at 16 μg/mL independent of the concentration of bis(3-aminopropyl)amine (0−64 μg/mL). Since enzymatic potency was still submicromolar for all these compounds, LPS electrophoresis assays were run to determine their ability to inhibit LPS formation in *E. coli* K1 Δ-*tolC* strain. For the difference of **41**, which displays almost complete LPS biosynthetic inhibition at 32 μg/mL, none of these polycationic compounds were able to inhibit LPS formation at this dose. This suggests that these compounds do not reach their active cytosolic concentration but are still capable of destabilizing the OM to allow translocation of erythromycin in *E. coli* in a

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nonspecific manner. Here again, one could speculate that this effect is probably induced by the amphiphilic character of the polycationic moieties linked to the lipophilic inhibitors, which may facilitate the permeation of erythromycin. Compound **18** was then designed with a balance of 8% of the dicationic form to destabilize the OM along with 6% of the uncharged form aiming at a better IM passage. The synergy results were under par, reinforcing the observation that a high amount of dicationic form is required for erythromycin synergy in this polycationic vectorization strategy.

CONCLUSION

We have described our efforts at vectorizing nanomolar inhibitors of HldE-kinase, a Gram-negative cytosolic target, using different strategies. Siderophoric vectorization led in a few cases to some improvement on efflux-pump deleted bacteria, possibly more related to the displacement of LPS-stabilizing cations than true siderophoric uptake. However, this strategy did not prove to be effective in wild bacteria, suggesting the need for a more complete siderophoric vector or some drug-release mechanism in the periplasm to better cross the IM. Aminoglycosidic vectorization afforded for the first time erythromycin synergy with our inhibitors in wild strains, and yet it was a nonspecific effect probably induced by the aminoglycoside displacement of the stabilizing dications of the LPS. Amphoteric vectorization proved to be disappointing in our examples because of the resulting loss of enzymatic activity that prevented an assessment of the real potential of this approach. Polycationic vectorization generated in quite a few instances erythromycin synergies with our inhibitors in wild bacteria. Like in the case of aminoglycosidic vectorization, this was achieved in a nonspecific manner probably induced by OM destabilization while the positive charges associated with the vector were unable to cross or destabilize the IM. Nevertheless, these polycationic examples provide interesting considerations about the required stereoelectronic arrangement of the amines and their basicity requirements to fulfill OM destabilization resulting in better erythromycin synergies. This work highlights the continuing challenge of finding suitable vectors to increase Gram-negative intracellular drug concentration.

EXPERIMENTAL SECTION

HldE-K Enzyme Assay. The assay buffer "AB" contained 50 mM Hepes, pH 7.5, 1 mM $MnCl_2$, 25 mM KCl, 0.012% Triton-X100, 1 mM dithiothreitol (DTT), and 0.1 μM myelin basic protein (MBP). The following components were added to a white polystyrene Costar plate to a final volume of 30 μL: 10 μL of inhibitor dissolved in DMSO/water, 50/50, and 20 μL of HldE of *E. coli* in AB. After 30 min of preincubation at room temperature, an amount of 30 μL of substrates mix in AB was added to each well to a final volume of 60 μL. This reaction mixture was then composed of 3 nM HldE, 0.2 μM β-heptose 7-phosphate (custom synthesis), and 0.2 μM ATP (Sigma) in assay buffer. After 30 min of incubation at room temperature, an amount of 200 μL of the mix was added to a final volume of 260 μL, including the following constituents at the respective final concentrations: 5000 light units/mL luciferase (Sigma), 30 μM D-luciferin (Sigma), 100 μM *N*-acetylcysteamine (Aldrich). Luminescence intensity was immediately measured on Luminoskan (Thermofischer) and converted into inhibition percentages. For IC_{50} determinations, the inhibitor was tested at 6−10 different concentrations, and the related inhibitions were fitted to a classical Langmuir equilibrium model using XLFIT (IDBS).

Antimicrobial Synergy with Erythromycin. Erythromycin is much more potent on *E. coli* Δ-*hldE* (MIC= 0.5−2 μg/mL) than on *E. coli* wild type strains (MIC = 32−64 μg/mL). The *hldE*-deleted strains are in fact more permeable to hydrophobic antibiotics because of their reduced LPS hydrophilic barrier; this is the basic principle of this simple assay. All chemicals were from Sigma unless otherwise stated. From early to log phase preculture, the bacterial suspension was prepared in ca-MHB (Becton-Dickinson) or M9 supplemented (M9S) medium to obtain a final inoculum of 5×10^5 CFU/mL. M9S was made of M9 medium supplemented with D-glucose (100 g/L), thiamine (10 g/L), casein (20 g/L), and apotransferrin (10 g/L). Strains were *E. coli* K12 MG1655 Δ-*acrAB*-Δ*tolc*, *E. coli* K1 (018:K1:H7) wild type, and Δ*tolc*. Growth conditions included erythromycin, or not, at respective concentrations of 1, 8, 1 μg/mL in ca-MHB and 0.13, 2, 0.25 μg/mL in M9S. The MIC of studied compounds were determined against these strains and conditions by visual inspection in polystyrene 96-well microplates containing serial dilutions of compounds in 2% DMSO in a final volume of 100 μL and after overnight incubation at 35 °C.

Checkerboard Assays. The effect of combining two compounds against *E. coli* K1 (018:K1:H7) was studied by checkerboard assay. From early to log phase preculture, the bacterial suspension was prepared in ca-MHB (Becton-Dickinson) to obtain a final inoculum of 5×10^5 CFU/mL. The combined serial dilutions contained kanamycin (Sigma, ref K4000) or compound **41** or bis(3-aminopropyl)amine (Aldrich ref I1006) with erythromycin (Fluka, ref 45674). The MICs of studied combinations were determined by visual inspection in polystyrene 96-well microplates in a final volume of 100 μL and 2% DMSO after overnight incubation at 35 °C.

Inhibition of *E. coli* K1 Wild Type or Δ*tolc* LPS Biosynthesis. *Principle.* *E. coli* K1 (018:K1:H7) is a newborn meningitidis *E. coli* (NMEC) strain that displays a typical LPS made of lipid A successively branched with the inner and outer core oligosaccharides and finally with the O-antigen repeats. The inner core contains several heptose residues. An inhibitor of the LPS heptosylation pathway should therefore reduce dramatically the size of LPS from full-length to the so-called "Re-LPS" limited to lipid A branched with two Kdo residues. A simple way of monitoring LPS size and composition consists of running LPS gel electrophoresis: a wild type *E. coli* strain displays several bands including those for full and core LPS but none for Re-LPS. On the contrary, a Δ-*hldE* mutant defective for LPS-heptosylation biosynthesis displays only the Re-LPS band.

Bacterial Culture. The effect of heptosylation inhibitors on *E. coli* LPS was assessed as described below. The compounds to be tested were prepared in deionized water/DMSO (50/50) solutions and added (25 μL) to sterile culture microtubes. The strains used in this study were *E. coli* K1 (018:K1:H7) wild type or Δ*tolc*. The bacteria were isolated on tryptic soy agar (TSA) overnight. Isolated colonies were cultured in 10 mL of Luria−Bertani medium (LB) at 37 °C to an optical density of typically 0.15. These exponentially growing bacteria were finally diluted to 5×10^5 CFU/mL and added to each well (225 μL) for incubation with the compounds at 37 °C for approximately 5 h, up to an optical density of 0.2−0.4.

LPS Extraction. Bacterial cultures were normalized via OD determination, pelleted, and washed with 1 mL of phosphate buffered saline (PBS). The pellets were then denatured for 10 min at 95−100 °C in 50 μL of sodium dodecyl sulfate 0.2% (SDS), β-mercaptoethanol 1%, glycerol 36%, Tris, pH 7.4, 30 mM, and bromophenol blue 0.001%. Samples were cooled to room temperature, supplemented with 1.5 μL of proteinase K at 20 mg/mL, incubated for 1 h at 55 °C, and centrifuged for 30 min at 13 000 rpm at 25 °C. The resulting supernatant containing LPS was finally analyzed by SDS−PAGE electrophoresis.

LPS SDS−PAGE Electrophoresis. Polyacrylamide gels (16% and 4% acrylamide for separation and concentration, respectively) were prepared, loaded with 8 μL of LPS extracts, and migrated.

Silver Staining. Gels were incubated overnight in 5% acetic acid/40% ethanol/deionized water, treated with 1% periodic acid/5% acetic acid for 15 min, washed 4 times for 10 min in deionized water, and finally incubated for 18 min in the dark in a silver nitrate solution composed of 56 mL of NaOH 0.1 N, 4 mL of ammoniac 33%, 45 mL of $AgNO_3$ 5% (Tsai and Frasch), and 195 mL of deionized water. Gels were then washed extensively in deionized water for 30 min and

incubated for 10−15 min (up to LPS bands apparition) in the mixture composed of 300 mL of deionized water, 300 μL of formaldehyde 36.5% (Fluka), and 100 μL of citric acid 2.3 M. The reaction was stopped by incubating the gels in acetic acid 10% for 5 min. Gels were finally washed in deionized water, numerized with a Samsung PL51 camera, and analyzed by ImageJ software. The percentage of inhibition of LPS heptosylation was defined as the relative area of the Re-LPS band compared to the cumulated areas of Re-LPS and Core-LPS bands.

General Experimental. All reactions were carried out under inert (nitrogen or argon) atmosphere unless indicated otherwise. Reagents and solvents were obtained from commercial sources and were used without further purification. Celite is a filter aid composed of diatomaceous silica and is a registered trademark of Celite Corporation. Analtech silica gel GF and E. Merck silica gel 60 F-254 thin layer plates were used for thin layer chromatography. Flash chromatography was carried out on Flashsmart Pack cartridge irregular silica 40−60 μm or spherical silica 20−40 μm. Preparative thin layer chromatography was carried out on Analtech silica gel GF 1000 μm 20 cm × 20 cm. Yields refer to purified products and are not optimized. All new compounds gave satisfactory analytical data. ^{1}H NMR spectra were recorded at 300 or 400 MHz on a Brüker instrument, and chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane. Mass spectra were obtained using electrospray (ESI) ionization techniques on an Agilent 1100 series LCMS instrument. HPLC (analytical and preparative) was performed on an Agilent 1100 HPLC instrument with diode array detection. Preparative HPLC was performed at 0.7 mL/min on a Thermo Electron, Hypersil BDS C-18 column (250 mm × 4.6 mm, 5 μm) using a gradient of acetonitrile and water with 0.1% TFA (50% in acetonitrile to 100% and then back to 50%). The tested compounds were determined to be >95% pure via HPLC.

2-{[2-({[5-(2,6-Dichlorophenyl)-1,2,4-triazin-3-yl]amino}methyl)-1,3-benzothiazol-5-yl]oxy}-*N*-hydroxyacetamide (1). To acid **19**[10] (55 mg, 0.119 mmol) were added 1-hydroxybenzotriazol (321 mg, 0.155 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (34 mg, 0.179 mmol), and dry dimethylformamide (1 mL). The mixture was stirred for 1 h at room temperature. Then to the mixture were added diisopropylethylamine (23 μL, 0.130 mmol) and hydroxylamine hydrochloride (9 mg, 0.130 mmol). The mixture was stirred overnight and then partitioned between saturated $NaHCO_3$ and ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (10% of methanol in dichloromethane) to afford **1** (21 mg, 37%). MS (ESI$^+$): 477 [M + H]$^+$. ^{1}H MNR (400 MHz, DMSO-d_6) δ: 10.88 (1H, s); 8.99 (1H, s); 8.87 (1H, s); 7.91 (1H, d, J = 8.6 Hz); 7.70−7.48 (4H, m); 7.09 (1H, d, J = 7.3 Hz); 5.10−4.8 (2H, m); 4.55 (2H, s).

(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)acetic Acid 1,5-Bis(4-methoxybenzyloxy)-4-oxo-1,4-dihydropyridin-2-ylmethyl Ester (21). To acid **19**[10] (50 mg, 0.11 mmol) were added 2-hydroxymethyl-1,5-bis(4-methoxybenzyloxy)-1*H*-pyridin-4-one **20**[27] (44 mg, 0.11 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (85 mg, 0.44 mmol), 4-dimethylaminopyridine (DMAP) (4 mg, 0.03 mmol), and dry THF (2 mL). The mixture was stirred for 15 h at room temperature, then concentrated under reduced pressure and then partitioned between water and ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (10% of methanol in dichloromethane) to afford compound **21** as a yellowish oil (30 mg, 32%). MS (ESI$^+$): 841, 843 [M + H]$^+$.

(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)acetic Acid 1,5-Dihydroxy-4-oxo-1,4-dihydropyridin-2-ylmethyl Ester (6). To a solution of compound **21** (28 mg, 0.033 mmol) in dichloromethane (1 mL) was added dropwise trifluoroacetic acid (TFA) (0.1 mL, 1.3 mmol), and the obtained mixture was stirred at room temperature overnight. Reaction mixture was then concentrated under reduced pressure to dry and was triturated with diethyl ether. The obtained yellow precipitate was then filtered, washed with MeOH (2 mL) and water (0.5 mL), then dried to afford **6** (9.2 mg, 46%) as a yellow powder. MS (ESI$^+$): 601, 603 [M + H]$^+$. ^{1}H MNR (400 MHz, DMSO-d_6) δ: 9.15 (1H, br s); 8.86 (1H, s); 7.94 (1H, d, J = 8.7 Hz); 7.75 (1H, s); 7.70−7.52 (4H, m); 7.11 (1H, d, J = 8.7 Hz); 6.81 (1H, s); 5.19 (2H, s); 5.12−4.96 (4H, m).

(2-*tert*-Butoxycarbonylaminoethyl)-{2-[2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)acetylamino]ethyl}carbamic Acid *tert*-Butyl Ester (22). To acid **19**[10] (40 mg, 0.060 mmol) were added 1-hydroxybenzotriazol (30 mg, 0.222 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (50 mg, 0.260 mmol), and dry dimethylformamide (0.5 mL). The mixture was stirred for 1 h at room temperature. Then to the mixture were added diisopropylethylamine (33 μL, 0.189 mmol) and 1,4-bis-Boc-1,4,7-triazaheptane (29 mg, 0.095 mmol). The mixture was stirred overnight and then partitioned between ice−water and ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (10% of methanol in dichloromethane) to afford **22** (36 mg, 56%) as a colorless oil. MS (ESI$^+$): 747−749 [M + H]$^+$.

***N*-[2-(2-Aminoethylamino)ethyl]-2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)acetamide (14).** To a solution of **22** (36 mg, 0.048 mmol) in 1.5 mL of dichloromethane−methanol (2:1) was added HCl 4 M in dioxane (300 μL, 1.20 mmol), and the mixture was stirred for 3 h at room temperature. The mixture was evaporated to dryness to afford **14** as a brown solid hydrochloride salt (34 mg, 63%). MS (ESI$^+$): 547 [M + H]$^+$. ^{1}H MNR (400 MHz, DMSO-d_6) δ: 9.36 (2H, br s); 8.86 (1H, s); 8.46 (1H, t, J = 5.5 Hz); 8.25 (3H, br s); 7.94 (1H, d, J = 8.8 Hz); 7.70−7.53 (4H, m); 7.17 (1H, dd, J_1 = 8.7 Hz, J_2 = 1.9 Hz); 5.03 (2H, br s); 4.62 (2H, s); 3.23−3.11 (6H, m).

***N*,*N*-Bis(3-aminopropyl)-2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]-triazin-3-ylamino]methyl}benzothiazol-5-yloxy)acetamide (15).** **15** was obtained from acid **19**[10] and [3-(3-*tert*-butoxycarbonylaminopropylamino)propyl]carbamic acid *tert*-butyl ester **32**[28] in similar conditions as described for **22** and **14**. MS (ESI$^+$): 575, 577 [M + H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.18 (1H, br s); 8.87 (1H, s); 8.11 (3H, m); 7.98−7.86 (4H, m), 7.72−7.53 (3H, m), 7.47 (1H, br s), 7.07 (1H, dd, J_1 = 8.7 Hz, J_2 = 2.1 Hz); 5.05 (2H, br s), 4.93 (2H, s), 3.44 (2H, t, J = 7.5 Hz); 3.37 (2H, t, J = 7.0 Hz); 2.86 (2H, m), 2.76 (2H, m), 1.94 (2H, quint, J = 7.3 Hz); 1.81 (2H, quint, J = 7.0 Hz).

***N*-{2-[Bis(2-aminoethyl)amino]ethyl}-2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)acetamide (16).** **16** was obtained from acid **19**[10] and {2-[(2-aminoethyl)-(2-*tert*-butoxycarbonylaminoethyl)amino]ethyl}carbamic acid *tert*-butyl ester[43] in similar conditions as described for **22** and **14**. MS (ESI$^+$): 590, 592 [M + H]$^+$. ^{1}H NMR (400 MHz, MeOD) δ ppm: 8.73 (1H, s); 7.87 (1H, d, J = 8.9 Hz); 7.51 (4H, br s); 7.23 (1H, dd, J_1 = 8.9 Hz, J_2 = 2.4 Hz); 5.11 (2H, br s); 4.70 (2H, s); 3.51 (2H, m); 3.35 (1H, s); 3.19−2.80 (11H, m).

2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]-methyl}benzothiazol-5-yloxy)-*N*-(2-dimethylamino-1-dimethylaminomethylethyl)acetamide (18). **18** was obtained from acid **19**[10] and *N*,*N*,*N*″,*N*″-tetramethylpropane-1,2,3-triamine[44] in similar conditions as described for **22** and **14**. MS (ESI$^+$): 589, 591 [M + H]$^+$. ^{1}H NMR (400 MHz, MeOD) δ ppm: 8.75 (1H, s); 7.91 (1H, d, J = 8.9 Hz); 7.55 (4H, m); 7.21 (1H, dd, J_1 = 8.9 Hz, J_2 = 2.4 Hz); 5.11 (2H, br s); 4.69 (2H, s); 3.59 (1H, dd, J_1 = 13.5 Hz, J_2 = 5.6 Hz); 3.38 (1H, dd, J_1 = 13.6 Hz, J_2 = 5.6 Hz); 2.95 (1H, m); 2.68 (1H, m); 2.52 (1H, m); 2.40 (12H, s).

{2-[2-({5-[2-Methyl-6-(tetrahydropyran-2-yloxy)phenyl]-[1,2,4]triazin-3-ylamino}methyl)benzothiazol-5-yloxy]ethyl}-carbamic Acid *tert*-Butyl Ester (25). To a mixture of 3-chloro-5-[2-methyl-6-(tetrahydropyran-2-yloxy)phenyl][1,2,4]triazine **23**[10] (150 mg, 0.48 mmol) in acetonitrile (2 mL) were added [2-(2-aminomethylbenzothiazol-5-yloxy)ethyl]carbamic acid *tert*-butyl ester **24**[10] (171 mg, 0.53 mmol) and DIPEA (125 μL, 0.72 mmol), and the mixture was stirred for 14 h at 85 °C. Reaction mixture was concentrated to dryness and then dissolved in EtOAc. The formed precipitate of the remaining starting compound **5** was filtered off and discarded. The filtrate was washed with water, and the organic layers

were combined, dried over Na_2SO_4, filtered, and concentrated. An oily residue was obtained and was purified by preparative TLC on silica gel (5% of methanol in dichloromethane) to afford compound **25** (128 mg, 45%). MS (ESI^+): 593 $[M + H]^+$.

2-(3-{[5-(2-Aminoethoxy)benzothiazol-2-ylmethyl]amino}-[1,2,4]triazin-5-yl)-3-methylphenol Hydrochloride (26). To a solution of **25** (128 mg, 0.21 mmol) in a mixture of dichloromethane (1 mL) and methanol (0.2 mL) was added dropwise a 4 M solution of HCl in dioxane (525 μL, 2.1 mmol) at 0 °C. The mixture was stirred for 4 h at room temperature. The mixture was evaporated to dryness to afford **26** as a white solid hydrochloride salt (53 mg, 62%). MS (ESI^+): 409 $[M + H]^+$.

4-Ethyl-2,3-dioxopiperazine-1-carboxylic Acid [2-(2-{[5-(2-Hydroxy-6-methylphenyl)[1,2,4]triazin-3-ylamino]methyl}-benzothiazol-5-yloxy)ethyl]amide (2). To the solution of **26** (30 mg, 0.073 mmol) in the mixture of THF (0.7 mL) and DMF (0.4 mL) were added DIPEA (39 μL, 0.22 mmol) and suspension of 4-ethyl-2,3-dioxopiperazine-1-carbonyl chloride (34 mg, 0.16 mmol) in 0.5 mL of THF at 0 °C under an argon atmosphere. The obtained mixture was then stirred at room temperature overnight. The mixture was evaporated to dryness, and the residue was partitioned between ice−water and ethyl acetate. The organic layer was dried over Na_2SO_4, filtered, and concentrated. The obtained oily residue was purified by preparative TLC on silica gel (8% of methanol in dichloromethane) to afford **2** as a yellow powder (12 mg, 29%). MS (ESI^+): 577 $[M + H]^+$. ^1H MNR (400 MHz, MeOD) δ: 8.74 (1H, s); 7.82 (1H, d, J = 8.8 Hz); 7.51 (1H, d, J = 2.3 Hz); 7.21 (1H, t, J = 7.8 Hz); 7.13 (1H, dd, J_1 = 8.8 Hz, J_2 = 2.3 Hz); 6.80 (2H, m); 5.10 (2H, s); 4.25 (2H, t, J = 5.3 Hz); 4.11 (2H, m); 3.82 (2H, t, J = 5.3 Hz); 3.67 (2H, m), 3.57 (2H, q, J = 7.2 Hz); 2.06 (3H, br s); 1.25 (3H, t, J = 7.2 Hz).

***N*-[2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]-methyl}benzothiazol-5-yloxy)ethyl]-3,4-dimethoxybenzene-sulfonamide (28).** To a solution of **27**[10] (hydrochloride salt, 61 mg, 0.128 mmol) and DIPEA (91 μL, 0.512 mmol) in a mixture of dioxane (1 mL) and DMF (1 mL) was added dropwise at 0 °C with stirring under argon freshly prepared solution of 3,4-dimethoxybenzenesulfonyl chloride in DMF (1 mL). Then the reaction mixture was allowed to heat to room temperature and was stirred at this temperature for 1 h. The reaction mixture was poured into ice (∼5 mL), extracted with ethyl acetate, and washed with saturated aqueous solution of NH_4Cl. The combined organic phases were dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (5% of methanol in dichloromethane) to afford compound **28** as white powder (64 mg, 78%). MS (ESI^+): 645, 647 $[M + H]^+$.

***N*-[2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]-methyl}benzothiazol-5-yloxy)ethyl]-3,4-dihydroxybenzenesulfonamide (3).** To a solution of **28** (26 mg, 0.045 mmol) in dichloromethane (1 mL) under argon atmosphere at −78 °C was added dropwise a 1 M solution of BBr_3 (1.12 mmol) in dichloromethane, and the mixture was allowed to warm from −78 to −30 °C over 2 h. Then the mixture was cooled to −50 °C, quenched by methanol (1 mL), and allowed to warm to room temperature overnight. The mixture was concentrated under reduced pressure, partitioned between water and ethyl acetate, and then additionally extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (6% of methanol in dichloromethane) to afford compound **3** as beige powder (5.8 mg, 20%). MS (ESI^+): 619, 621 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ: 8.75 (1H, s); 7.81 (1H, d, J = 8.8 Hz); 7.55 (3H, m); 7.41 (1H, br s); 7.33 (1H, d, J = 2.2 Hz); 7.30 (1H, dd, J_1 = 8.3 Hz, J_2 = 2.2 Hz); 7.05 (1H, dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz); 6.90 (1H, d, J = 8.3 Hz); 5.10 (2H, br s); 4.10 (2H, t, J = 5.5 Hz); 3.34 (2H, t, J = 5.5 Hz).

(*R*)-2-(2-Hydroxyphenyl)-4,5-dihydrothiazole-4-carboxylic Acid [2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]-methyl}benzothiazol-5-yloxy)ethyl]amide (8). To a thin suspension of **27**[10] (hydrochloride salt, 57 mg, 0.127 mmol) and DIPEA (44 μL, 0.25 mmol) in dichloromethane (1 mL) were added (*R*)-2-(2-hydroxyphenyl)-4,5-dihydro-1,3-thiazole-4-carboxylic acid[45] (33 mg, 0.15 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 48 mg, 0.25 mmol), 1-hydroxybenzotriazol hydrate (20 mg, 0.128 mmol), and 4-dimethylaminopyridine (2 mg, cat.) under argon atmosphere. The mixture was sonicated and then was stirred at room temperature overnight, then concentrated under reduced pressure, partitioned between water and ethyl acetate, and then additionally extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (5% of methanol in dichloromethane) to afford compound **8** as an off-white powder (25 mg, 30%). MS (ESI^+): 652, 654 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ: 12.11 (1H, br s); 9.20 (1H, br s); 8.87 (1H, s); 8.57 (1H, br s); 7.89 (1H, d, J = 8.5 Hz); 7.75−7.38 (6H, m); 7.05 (1H, d, J = 8.5 Hz); 6.98 (2H, m); 5.35 (1H, t, J = 8.9 Hz); 5.04 (2H, br s); 4.14 (2H, t, J = 5.2 Hz); 3.69−3.55 (4H, m).

5-Hydroxy-4-oxo-4*H*-pyran-2-carboxylic Acid [2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)ethyl]amide (5). MS (ESI^+): 585, 587 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ: 9.56 (1H, br s); 9.11 (1H, t, J = 5.3 Hz); 8.86 (1H, s); 8.12 (1H, s); 7.90 (1H, d, J = 8.6 Hz); 7.72−7.46 (5H, m); 7.05 (1H, d, J = 7.0 Hz); 6.90 (1H, s); 5.20−4.80 (2H, m); 4.19 (2H, t, J = 5.1 Hz); 3.65 (2H, q, J = 5.4 Hz).

Methanesulfonic Acid 2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]-triazin-3-ylamino]methyl}benzothiazol-5-yloxy)ethyl Ester (30). To a solution of 2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)ethanol **29**[10] (80 mg, 0.18 mmol) and triethylamine (28 μL, 0.2 mmol) in anhydrous THF (2 mL) at 0 C° under argon atmosphere was added dropwise mesyl chloride (16 μL, 0.2 mmol). The mixture was stirred at 0 °C for 30 min and thereafter allowed to warm to room temperature over 2 h. After concentration in vacuo and treatment with water and ethyl acetate, the combined organic layers were washed with 0.05 M HCl, $NaHCO_3$, brine, dried over Na_2SO_4, and filtered. After concentration, the yellowish oil product **30** (85 mg, 90% crude yield) was used as such without further purification. MS (ESI^+): 526, 528 $[M + H]^+$.

3-[2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]-methyl}benzothiazol-5-yloxy)ethylsulfanyl]-6-hydroxy-2-methyl-2*H*-[1,2,4]triazin-5-one (7). A mixture of 1,2,5,6-tetrahydro-5,6-dioxo-3-mercapto-2-methyl-as-triazine (44 mg, 0.28 mmol) and triethylamine (20 μL, 0.14 mmol) in DMF (2 mL) was added to freshly prepared **30** (47 mg, 0.09 mmol), and the obtained solution was stirred at 60 °C for 72 h. Then the mixture was treated with ice−water and ethyl acetate and extracted additionally with ethyl acetate, dichloromethane, and butanol. The combined organic layers were dried over Na_2SO_4 and filtered. The filtrate was concentrated, and the residue was purified by preparative TLC on silica gel (6% of methanol in dichloromethane) to afford compound **7** as a beige powder (15 mg, 28%). MS (ESI^+): 589, 591 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ: 13.18 (1H, br s); 9.15 (1H, br s); 8.86 (1H, s); 7.91 (1H, d, J = 8.4 Hz); 7.75−7.51 (4H, m); 7.09 (1H, d, J = 8.4 Hz); 5.04 (2H, br s); 4.45 (2H, t, J = 5.3 Hz); 4.41 (2H, t, J = 5.3 Hz), 3.72 (3H, s).

(3-{(3-*tert*-Butoxycarbonylaminopropyl)[2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)ethyl]amino}propyl)carbamic Acid *tert*-Butyl Ester (31). A mixtute of [3-(3-*tert*-butoxycarbonylaminopropylamino)-propyl]carbamic acid *tert*-butyl ester **32**[28] (58 mg, 0.18 mmol) and **30** (47 mg, 0.09 mmol) in acetonitrile (0.5 mL) was stirred at 85 °C for 48 h. The mixture was concentrated in vacuo and treated with water and ethyl acetate. The separated organic layer was washed with a saturated aqueous solution of NH_4Cl, dried over Na_2SO_4, and filtered. The filtrate was concentrated and the residue was purified by preparative TLC on silica gel (33% aqueous NH_3/methanol/dichloromethane 0.7/7/93) to afford compound **31** as a colorless oil (30 mg, 43%). MS (ESI^+): 761, 763 $[M + H]^+$.

***N*^{1}-(3-Aminopropyl)-*N*^{1}-[2-(2-{[5-(2,6-dichlorophenyl)[1,2,4]-triazin-3-ylamino]methyl}benzothiazol-5-yloxy)ethyl]-propane-1,3-diamine (17).** To a solution of **31** (30 mg, 0.39 mmol) in dichloromethane (0.7 mL) was added 4 N solution of HCl in dioxane (0.3 mL, 1.2 mmol) at 0 °C under argon atmosphere, and the mixture was stirred at room temperature for 3 h. The obtained hydrochloride was desalted by purification through Agilent SCX cartridge, using DCM, then MeOH, then 1 M ammonia solution in MeOH as eluents, affording **17** as a colorless oil (5.1 mg, 23%). MS

(ESI$^+$): 561, 563 [M + H]$^+$. ^{1}H NMR (400 MHz, MeOD) δ: 8.74 (1H, s); 7.83 (1H, d, J = 8.8 Hz); 7.59−7.48 (4H, m); 7.12 (1H, dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz); 5.11 (2H, br s); 4.23 (2H, t, J = 5.5 Hz); 2.99 (2H, t, J = 5.5 Hz); 2.75 (4H, t, J = 7.0 Hz); 2.69 (4H, t, J = 7.1 Hz); 1.74 (4H, quint, J = 7.1 Hz).

[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-yl]-{5-[2-(4-methylpiperazin-1-yl)ethoxy]benzothiazol-2-ylmethyl}amine (13). MS (ESI$^+$): 530, 532 [M + H]$^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.70 (1H, s); 7.68 (1H, d, J = 8.8 Hz); 7.45 (2H, dd, J_1 = 11.8 Hz, J_2 = 10.3 Hz); 7.42 (1H, s); 7.35 (1H, dd, J_1 = 9.1 Hz, J_2 = 6.8 Hz); 7.04 (1H, dd, J_1 = 8.8 Hz, J_2 = 6.4 Hz); 6.50−6.25 (1H, br s); 5.22−5.12 (2H, br s); 4.18 (2H, t, J = 5.8 Hz); 2.86 (2H, t, J = 5.7 Hz); 2.72−2.56 (4H, br s); 2.56−2.40 (4H, br s); 2.30 (3H, s).

2-(2-{[5-(2,6-Dichlorophenyl)[1,2,4]triazin-3-ylamino]-methyl}benzothiazol-5-yloxy)-*N*-prop-2-ynylacetamide (34). To acid **19**[10] (100 mg, 0.22 mmol) were added 1-hydroxybenzotriazol (34 mg, 0.22 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (85 mg, 0.44 mmol), and dry dichloromethane (1 mL). The mixture was stirred for 1 h at room temperature. Then to the mixture were added diisopropylethylamine (78 μL, 0.44 mmol) and propargylamine (24 mg, 0.44 mmol). The mixture was stirred overnight at room temperature and then partitioned between water and ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated. The residue was purified by preparative TLC on silica gel (5% of methanol in dichloromethane) to afford **34** (48 mg, 44%) as a white powder. MS (ESI$^+$): 499, 501 [M + H]$^+$.

***N*,*N*′-[(1*R*,3*S*,4*R*,5*R*,6*S*)-4-({6-Deoxy-6-[(2,2-dimethylpropanoyl)amino]-α-D-glucopyranosyl}oxy)-6-({3,6-dideoxy-6-(4-{[({[2-({[5-(2,6-dichlorophenyl)-1,2,4-triazin-3-yl]-amino}methyl)-1,3-benzothiazol-5-yl]oxy}acetyl)amino]-methyl}-1*H*-1,2,3-triazol-1-yl)-3-[(2,2-dimethylpropanoyl)-amino]-α-D-glucopyranosyl}oxy)-5-hydroxycyclohexane-1,3-diyl]bis(2,2-dimethylpropanamide) (35).** In an amber flask N-protected 6″-azido-kanamycin A **33**[29] (80 mg, 0.088 mmol) and alkyne **34** (44 mg, 0.088 mmol) were diluted in *tert*-butanol (0.5 mL). To this solution were added a solution of copper sulfate (0.14 mg, 0.0009 mmol) and sodium ascorbate (2 mg, 0.009 mmol) in water (0.5 mL). Then the mixture was stirred for 3 days at room temperature. The mixture was partitioned between water and ethyl acetate. The aqueous layer was extracted with ethyl acetate. Then the combined organic layers were dried over Na_2SO_4 and concentrated in vacuum. The crude was purified by chromatography on silica gel (gradient 3−15% of methanol in dichloromethane) to afford compound **35** (15.4 mg, 12%) as a yellow solid. MS (ESI$^+$): 1408−1410 [M + H]$^+$. ^{1}H MNR (400 MHz, MeOD) δ: 8.69 (1H, s); 7.85−7.83 (2H, m); 7.50 (4H, br s); 7.19 (1H, dd,, J_1 = 8.8 Hz, J_2 = 6.4 Hz); 6.7−6.4 (3H, m); 5.17−4.95(4H, m); 4.75−4.35 (7H, m); 3.75−3.00 (18H, m); 3.00 (1H, br s); 1.48−1.38 (36H, m).

(1*S*,2*R*,3*R*,4*S*,6*R*)-4,6-Diamino-3-[(6-amino-3-[(6-amino-6-deoxy-α-D-glucopyranosyl)oxy]-2-hydroxycyclohexyl-3-amino-3,6-dideoxy-6-(4-{[({[2-({[5-(2,6-dichlorophenyl)-1,2,4-triazin-3-yl]amino}methyl)-1,3-benzothiazol-5-yl]oxy}acetyl)-amino]methyl}-1*H*-1,2,3-triazol-1-yl)-α-D-glucopyranoside (9). Compound **35** (15 mg, 0.010 mmol) was dissolved in methanol (0.5 mL), and HCl 4 M in dioxane (106 μL, 0.424 mmol) was added. The mixture was stirred overnight at room temperature and then concentrated in vacuo. The crude was dissolved in methanol (0.4 mL) and precipitated with diethyl ether (2 mL). The solid was isolated by filtration, washed with ether, and dried in vacuum. Compound **9** (7.8 mg, 64%) was obtained as a light yellow solid. MS (ESI$^+$): 1008−1010 [M + H]$^+$. ^{1}H MNR (400 MHz, MeOD) δ: 8.70 (1H, s); 7.90−7.75 (2H, m); 7.60−7.44 (4H, m); 7.21 (1H, d, J = 8.2 Hz); 5.56−5.46 (2H, m); 5.16−5.06 (3H, m); 4.64−3.36 (20H, m); 3.14−3.02 (2H, m); 2.47 (1H, s); 1.97 (1H, s).

2-({5-[4-(2-Dimethylaminoethylamino)-2,6-dimethylphenyl]-[1,2,4]triazin-3-ylamino}methyl)benzothiazol-5-ol (10). Compound **36**[10] (10.9 mg, 0.023 mmol) was dissolved in dichloromethane (0.5 mL) and cooled to −78 °C. Boron tribromide 1 M in dichloromethane (253 μL, 0.253 mmol) was added dropwise, and the mixture was stirred for 1 h at this temperature and then for 4 h at room temperature. The mixture was cooled to −78 °C and quenched with methanol (1 mL). The solution was diluted with saturated $NaHCO_3$ aqueous solution (5 mL) and extracted with ethyl acetate (3 × 2 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated in vacuum and the crude was purified by preparative TLC on silica gel (15% of methanol in dichloromethane) to give compound **10** (4.6 mg, 44%) as a yellow solid. MS (ESI$^+$): 450 [M + H]$^+$. ^{1}H MNR (400 MHz, MeOD) δ: 8.52 (1H, s); 7.68(1H, d, J =8.7 Hz); 7.29 (1H, d, J = 2.1 Hz); 6.92 (1H, dd,, J_1 = 8.7 Hz, J_2 = 2.4 Hz); 6.38 (1H, s); 5.00 (2H, br s); 2.79 (2H, t, J = 6.3 Hz); 2.48 (6H, s); 2.00−1.90 (6H, m).

2-Dimethylamino-*N*-(4-{3-[(5-hydroxybenzothiazol-2-ylmethyl)amino][1,2,4]triazin-5-yl}-3,5-dimethylphenyl)-acetamide (11). MS (ESI$^+$): 464 [M + H]$^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 9.70 (1H, br s); 9.64 (1H, s); 9.00−8.50 (2H, m); 7.76 (1H, d, J = 8.7 Hz); 7.43 (2H, br s); 7.25 (1H, s); 6.88 (1H, dd, J_1 = 8.9 Hz, J_2 = 6.6 Hz); 4.90 (2H, br s); 3.06 (2H, s); 2.27 (6H, s); 2.10−1.80 (6H; m).

***N*-[4-(3-{[5-(2-Aminoethoxy)benzothiazol-2-ylmethyl]-amino}[1,2,4]triazin-5-yl)-3,5-dimethylphenyl]-*N*′,*N*′-dimethyl-ethane-1,2-diamine (12).** (a) A solution of [4-(3-chloro[1,2,4]-triazin-5-yl)-3,5-dimethylphenyl]carbamic acid *tert*-butyl ester **38** (160 mg, 0.48 mmol),[10] *tert*-butyl 2-{[2-(aminomethyl)-1,3-benzothiazol-5-yl]oxy}ethylcarbamate **24** (180 mg, 0.56 mmol),[10] and diisopropylethylamine (84 μL, 0.48 mmol) in anhydrous acetonitrile (2 mL) was stirred under argon at 85 °C overnight. An aqueous solution of ammonium chloride was added, and the reaction mixture was extracted with ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered, and evaporated. Purification by preparative TLC (silica gel, dichloromethane/methanol 95/5) afforded *tert*-butyl [4-(3-{[5-(2-*tert*-butoxycarbonylaminoethoxy)benzothiazol-2-ylmethyl]amino}[1,2,4]triazin-5-yl)-3,5-dimethylphenyl]carbamate (174.1 mg, 58%) as an orange solid. ESI-MS m/z 622 (M + H)$^+$.

(b) (i) A mixture of 37% aqueous hydrochloric acid (1.25 mL) and ethyl acetate (3.75 mL) was added to *tert*-butyl [4-(3-{[5-(2-*tert*-butoxycarbonylaminoethoxy)benzothiazol-2-ylmethyl]amino}[1,2,4]-triazin-5-yl)-3,5-dimethylphenyl]carbamate (174.1 mg, 0.28 mmol), and the mixture was cooled to 0 °C. The reaction mixture was stirred for 1 h allowing the temperature to rise to room temperature. A dilute aqueous solution of potassium hydroxyde was added until basic pH was reached. The solution was extracted with ethyl acetate and dichloromethane, and the combined organic extracts were dried over sodium sulfate, filtered, and evaporated. (ii) The crude product was dissolved in anhydrous THF (3 mL), and triethylamine (60 μL, 0.43 mmol) and di-*tert*-butyl dicarbonate (67.2 mg, 0.31 mmol) were added. The resulting mixture was stirred at room temperature overnight. An aqueous solution of ammonium chloride was added, and the reaction mixture was extracted with diethyl ether and ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered, and evaporated. Purification by preparative TLC (silica gel, dichloromethane/methanol 93/7) afforded *tert*-butyl [2-(2-{[5-(4-amino-2,6-dimethylphenyl)[1,2,4]triazin-3-ylamino]methyl}-benzothiazol-5-yloxy)ethyl]carbamate (107 mg, 73%) as a yellow solid. ESI-MS m/z 522 (M + H)+.

(c) (i) Under argon, 2-chloro-*N*,*N*-dimethylethanamine hydrochloride (59 mg, 0.41 mmol), sodium hydrogencarbonate (34.5 mg, 0.41 mmol), and a catalytic amount of sodium iodide were successively added to a solution of *tert*-butyl [2-(2-{[5-(4-amino-2,6-dimethylphenyl)[1,2,4]triazin-3-ylamino]methyl}benzothiazol-5-yloxy)ethyl]carbamate (107 mg, 0.21 mmol) in ethanol (1.65 mL). The reaction mixture was stirred at room temperature for 1 h and then at 80 °C overnight. The solution was then dried over potassium carbonate, filtered, and concentrated. Purification by preparative TLC (silica gel, dichloromethane/methanol 85/15) led to {2-[2-({5-[4-(2-dimethylaminoethylamino)-2,6-dimethylphenyl][1,2,4]triazin-3-ylamino}methyl)benzothiazol-5-yloxy]ethyl}carbamic acid *tert*-butyl ester. (ii) The latter compound was dissolved in methanol (125 μL), and a solution of hydrogen chloride in dioxane (4 N, 375 μL, 1.5 mmol) was added. The resulting mixture was stirred at room temperature for 25 min. Methanol and dichloromethane were added, and the solution was dried over potassium carbonate, filtered, and

concentrated. The crude product was purified by preparative TLC (silica gel, dichloromethane/methanol/aqueous ammonium hydroxide 33%, 90/10/2) to afford compound **12** (13.6 mg, 13%) as a yellow solid. ESI-MS m/z 493 $(M + H)^+$. ^{1}H NMR (DMSO-d_6), δ (ppm): 8.63 (s, 1H), 7.91 (d, J = 8.8 Hz, 1H), 7.51 (d, J = 2.0 Hz, 1H), 7.07 (dd, J = 8.8 and 2.0 Hz, 1H), 6.32 (br s, 2H), 4.93 (br s, 2H), 4.16−4.13 (m, 2H), 3.12−3.10 (m, 4H), 2.44−2.40 (m, 2H), 2.18 (s, 6H), 1.91 (br s, 6H).

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Notes

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