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Structure-activity relationships of novel tryptamine-based inhibitors of bacterial transglycosylase

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Abstract

Penicillin-binding proteins represent well-established, validated and still very promising targets for the design and development of new antibacterial agents. The transglycosylase domain of penicillin-binding proteins is especially important, as it catalyzes polymerization of glycan chains, using the peptidoglycan precursor lipid II as a substrate. Based on the previous discovery of a non-covalent small-molecule inhibitor of transglycosylase activity, we systematically explored the structure-activity relationships of these tryptamine-based inhibitors. The main aim was to reduce the non-specific cytotoxic properties of the initial hit compound, and concurrently to retain the mode of its inhibition. A focused library of tryptamine-based compounds was synthesized, characterized, and evaluated biochemically. The results presented here show the successful reduction of the non-specific cytotoxicity, and the retention of the inhibition of transglycosylase enzymatic activity, as well as the ability of these compounds to bind to lipid II and to have antibacterial actions.

1. Introduction

The rapid development of antibacterial agents in the second half of the 20th century has resulted in remarkable advances for humanity. The rate of mortality from infectious diseases has decreased dramatically, and a perception of the final triumph over bacteria has spread to the public. However, this perception is erroneous, as the current increasing occurrence of resistance to existing antibiotics and the lack of novel antimicrobials under development are of great concern. The incidence of so-called 'superbugs' (i.e., organisms that are resistant to most of the clinically used antibiotics) is rapidly increasing, so new antibiotic drugs that exploit new or so-far-underexplored targets need to be identified to address this problem.[1–3] In the battle against the antibiotic crisis, alternative therapeutic concepts, such as inhibition of bacterial virulence and resistance factors, are also being pursued. Of these, different anti-adhesion and anti-biofilm strategies show promise and are receiving a lot of attention. The advantages, disadvantages, and future prospects of these antibacterial approaches were described recently.[4,5]

Peptidoglycan is the major component of the bacterial cell wall. Its polymer-like structure protects bacterial cells from rupture in challenging environments. Unobstructed synthesis of peptidoglycan is required for cell viability, and its inhibition leads to cell death. Many enzymes are involved in the synthesis of peptidoglycan, and most of these are potential targets for antibacterial agents.[6] The last two steps of peptidoglycan biosynthesis are particularly attractive for potential antibacterial compounds, as they take place on the external surface of the cytoplasmic membrane, and are therefore more accessible to inhibitors, at least in Gram-positive bacteria.

These transglycosylation and transpeptidation reactions are catalyzed by bifunctional penicillin-binding proteins (PBPs) that have transglycosylase (TG) and transpeptidase (TP) active sites.[7] In the transglycosylation step, the transfer of the sugar moiety from the activated polymeric peptidoglycan (a glycosyl donor) to the specific 4-hydroxyl group of lipid II (a glycosyl receptor) is achieved, with the concomitant release of a C55 undecaprenyl pyrophosphate carrier.[8,9] In the subsequent step, transpeptidation occurs, which produces inter-strand peptide cross-links. Several types of PBPs are present in bacteria, which belong to either bifunctional PBPs (class A) or monofunctional TP PBPs (class B). Some bacteria also have monofunctional TGs that form polysaccharide chains, which are then cross-linked by other PBPs.[7]

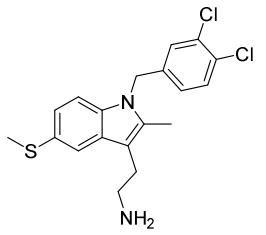
Most of the antibiotics that tackle peptidoglycan biosynthesis target the peptide cross-linking step by inhibiting the TP activity of PBPs directly (e.g., β-lactams), or by forming a complex with the D-Ala-D-Ala residue on the lipid II pentapeptide stem (e.g., vancomycin).[10] The TG activity has been traditionally viewed as very difficult to target, mostly due to the flat and extended active site topology.[11] It has been established that inhibition of the transglycosylation reaction and, consequently, the disruption of peptidoglycan assembly, can be accomplished either by compounds binding to the enzyme active site directly (enzyme binders) or by sequestration of lipid II (substrate binders), thus preventing its use as a substrate.[12] A prime example of a substrate binder is nisin, a naturally occurring lantibiotic that binds to lipid II via the pyrophosphate moiety, which induces pore formation in the bacterial membrane. The most extensively investigated TG enzyme binders are moenomycin and its analogs, as well as lipid II-based derivatives.

Recently, significant efforts from academia and industry resulted in the discovery of a few structurally diverse, low-molecular-weight and non-carbohydrate classes of TG inhibitors.[13] Moenomycin still remains the most potent inhibitor of TGs; unfortunately, despite intensive research, the greatest disadvantages of this natural product reside in its poor pharmacokinetic properties, which prevent moenomycin from being clinically useful in humans.[14] Therefore, the discovery of novel compounds with distinct structural features and non-carbohydrate scaffolds that can inhibit the TG activity are needed, and could lead to novel antibacterial drugs.

Recently, we described a hit compound 2-(1-(3,4-dichlorobenzyl)-2-methyl-5-(methylthio)-1*H*-indol-3-yl)ethanamine (hit compound; Figure 1) and showed that it interacts with the lipid II substrate via the pyrophosphate motif and inhibits TGs from different bacteria at low micromolar concentrations. In addition, the initial hit showed antibacterial activity, with MICs ranging from 4-16 μg/mL.[15] This compound, however, has several drawbacks; namely, troublesome synthesis, chemical instability (towards oxidation) due to the presence of the aromatic methylthio (SMe) moiety, and more importantly, non-specific cytotoxic characteristics (e.g., hit compound is severely cytotoxic on human embryonic kidney [HEK] 293 cells at 8 μg/mL).

Based on previous data[15] and cell viability results, we postulated that the initial hit compound exerts its antibacterial action via two mechanisms: specifically through the inhibition of cell-wall synthesis, and non-specifically through membrane disruption. Therefore, our main aim in terms of the optimization of the characteristics of this compound class was to eliminate the structural features that lead to the cytotoxic effects. Concurrently, we wanted to retain the TG inhibitory potency of new derivatives, and their ability to bind to lipid II. In this paper, we report on the design and synthesis of a focused library of tryptamine-based derivatives that were used to explore the structure-activity relationships (SARs) for inhibition of the TG activity. Our efforts

have resulted in a small set of novel and potent inhibitors of this very promising target that have antibacterial activity and very low non-specific cytotoxic effects.



hit compound

IC_{50} (*E. coli* PBP1b, μM) = 29 ± 12
IC_{50} (*S. aureus* PBP2, μM) = 20.7 ± 1
IC_{50} (*T. maritima* PBP1a, μM) = 56 ± 7
IC_{50} (*E. hirae* PBP2, μM) = 33 ± 6
IC_{50} (*S. aureus* MtgA, μM) = 27 ± 9

Figure 1. Previously published tryptamine-based hit compound (obtained from the National Cancer Institute) with potent activity against various TGs, and with MICs ranging from 4-16 μg/mL.[15]

2. Design and Chemistry

To establish the SARs of tryptamine-based derivatives for inhibition of TG activity and elimination of non-specific toxic effects, several approaches were pursued (Figure 2). We decided to retain the tryptamine core (Figure 2, green) and systematically modify the substituents of the parent compound. First, incorporation of a guanidine moiety instead of the amine was chosen based on the results of docking studies.[15] The initial hit compound appears to form an ionic bond with Asp156 and with the pyrophosphate group of lipid II. Therefore, we expected to boost the interactions with guanidine, as this might form additional H-bonds (besides the ionic one) with the pyrophosphate and Asp156 when compared to primary amine, and consequently improve the inhibitory potency. Next, we explored the role of different substituents on the benzyl ring attached to the nitrogen of the indole. Thirdly, we wanted to determine the importance of the

methyl group at position 2 of the indole for inhibition of TG activity. Finally, to omit the SMe group, we synthesized several compounds with a methoxy (OMe) substituent at position 5 of the indole, and a set of compounds with a 2,5-unsubstituted tryptamine scaffold.

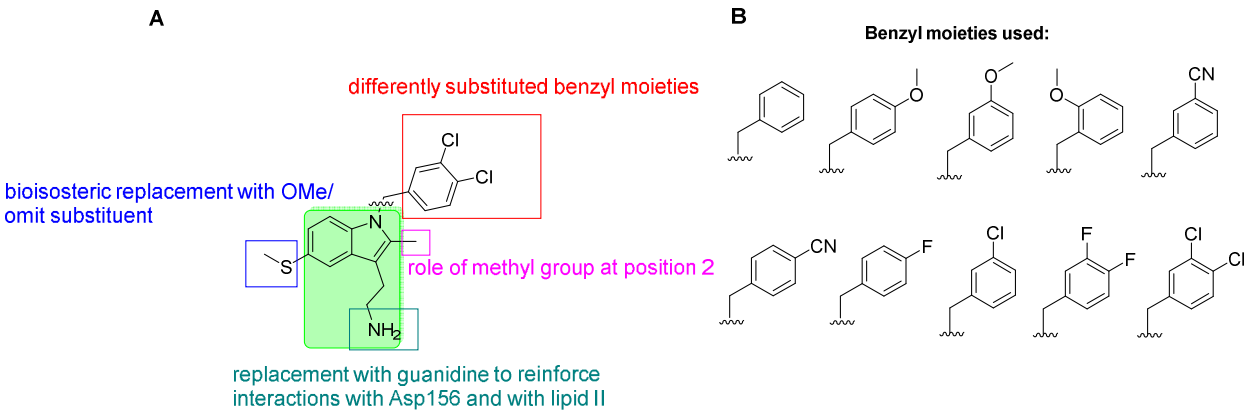


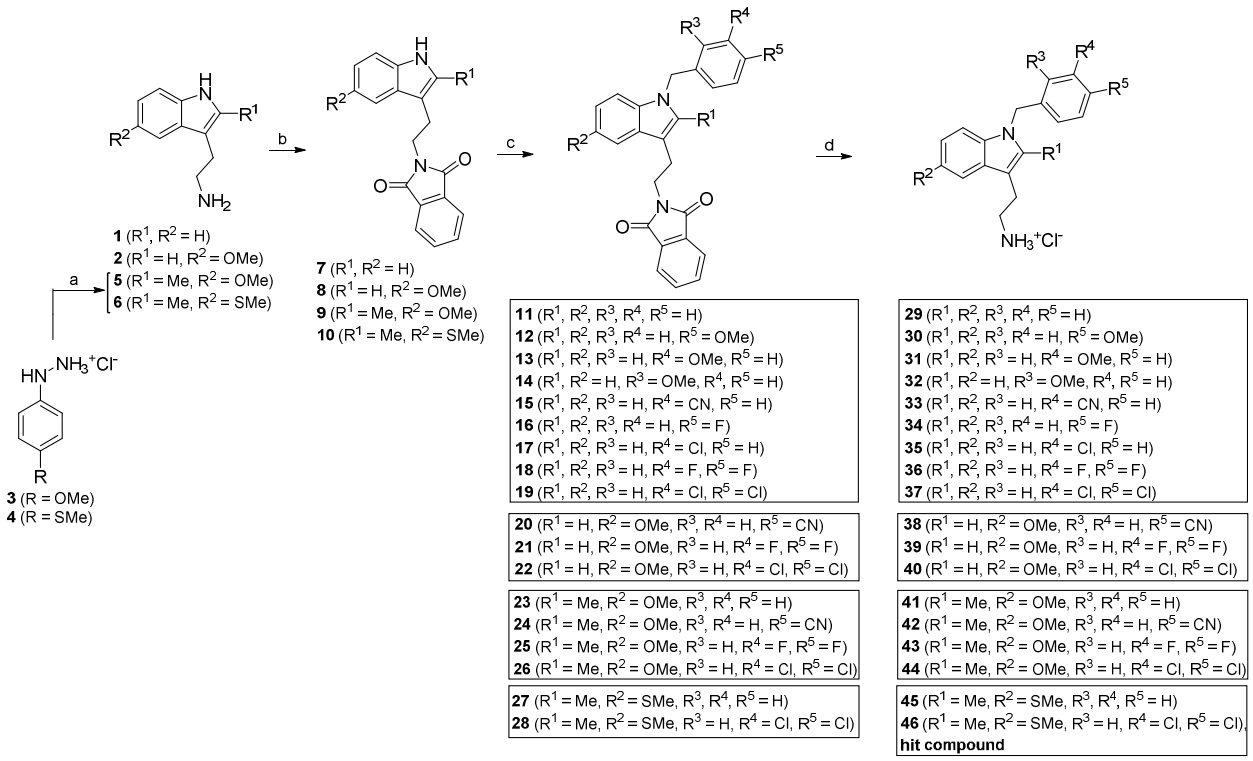
Figure 2. (A) Schematic representation of the systematic structural modifications to the hit compound. The tryptamine core is highlighted in green. (B) The benzyl moieties used in the SAR studies.

To accurately explore the chemical space and include different patterns of scaffold substitution, two tryptamine derivatives that were commercially available (**1** and **2**, Scheme 1) and two that needed to be synthesized (**5** and **6**, Scheme 1) were used as the core framework. The 2-methyl-5-methoxytryptamine (**5**) and 2-methyl-5-methylthiotryptamine (**6**) were prepared according to the previously described Grandberg indole synthesis.[16] Briefly, equimolar amounts of 4-methoxyphenylhydrazine or 4-thiomethylphenylhydrazine (both obtained from the corresponding hydrochloride salts **3** and **4** prior to the reaction) and 5-chloro-2-pentanone (technical grade, 85%) were mixed for 24 h in EtOH. Due to the exothermic nature of the initial

reaction, the temperature of the mixture during the addition of 5-chloropentan-2-one needed to be carefully monitored and kept below 40 °C (Scheme 1).

All of the tryptamine derivatives **1, 2**, **5**, and **6** were then subjected to phthalimide formation of the primary amine, by refluxing with phthalic anhydride in toluene using a Dean-Stark apparatus. Indole nitrogen benzylation of the phthaloyl-protected tryptamines **7**-**10** was achieved by treating these compounds with NaH or Cs_2CO_3 in DMF or CH_3CN, followed by alkylation with various benzyl bromides, to yield benzylated tryptamines **11**-**28** (Scheme 1). This three-step reaction sequence was completed with the deprotection of the primary amino group by heating the *N*-phthaloyl-protected compounds **11**-**28** with excess hydrazine in refluxing EtOH. Finally, these deprotected amines were converted into their corresponding hydrochloride salts, which yielded compounds **29**-**46** (Scheme 1). In addition, the hit compound was resynthesized and transformed into its hydrochloride form (compound **46**), to improve the chemical stability in comparison with the free aliphatic amine. Thus, a direct comparison of biological activities between compounds was possible (*see* Results and Discussion).

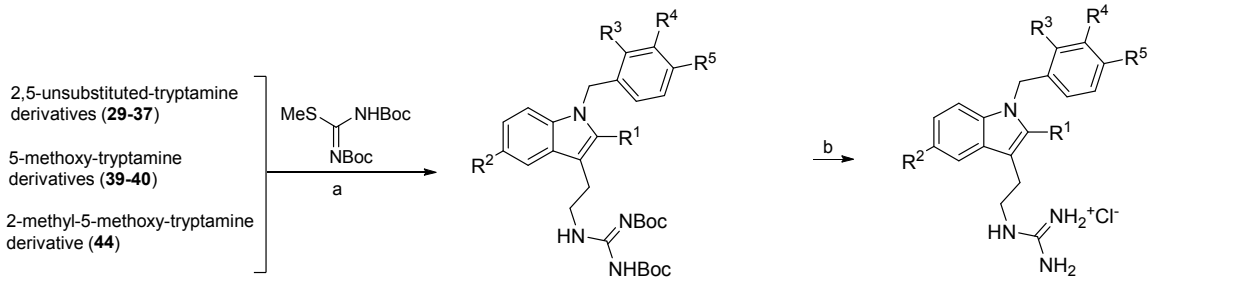
Scheme 1. Synthesis of the compounds with various substitution patterns on the tryptamine core[a].



[a] Reagents and conditions: (a) i. 2 M NaOH, H_2O; ii. 5-chloropentan-2-one, EtOH, 35 °C, heat to 85 °C, 24 h; (b) phthalic anhydride, Et_3N, toluene, reflux, 24 h; (c) for compounds **11**-**22**: corresponding benzyl bromide (Figure 2B), NaH, DMF, 55 °C, overnight; for compounds **23**-**28**: corresponding benzyl bromide (Figure 2B), Cs_2CO_3, CH_3CN, 80 °C, overnight; (d) i. $H_2NNH_2 \times H_2O$, EtOH, reflux, 24 h; ii. 2 M ethanolic HCl, EtOH.

The conversion of the amino moiety into guanidine was performed by applying a previously described two-step process,[17] in which the amines of a set of desired compounds were initially converted into the bis-Boc protected guanidines **47**-**58** using *N*,*N*'-di-(*tert*-butoxycarbonyl)-*S*-methylisothiourea in the presence of $HgCl_2$ (Scheme 2). This was followed by CF_3COOH-mediated Boc deprotection, and then conversion of the final compounds into the corresponding hydrochlorides **59**-**70** (Scheme 2).

Scheme 2. Synthesis of the compounds with the guanidine moiety[a].



47 (R^1, R^2, R^3, R^4, R^5 = H)
48 (R^1, R^2, R^3, R^4 = H, R^5 = OMe)
49 (R^1, R^2, R^3 = H, R^4 = OMe, R^5 = H)
50 (R^1, R^2 = H, R^3 = OMe, R^4, R^5 = H)
51 (R^1, R^2, R^3 = H, R^4 = CN, R^5 = H)
52 (R^1, R^2, R^3, R^4 = H, R^5 = F)
53 (R^1, R^2, R^3 = H, R^4 = Cl, R^5 = H)
54 (R^1, R^2, R^3 = H, R^4 = F, R^5 = F)
55 (R^1, R^2, R^3 = H, R^4 = Cl, R^5 = Cl)

56 (R^1 = H, R^2 = OMe, R^3 = H, R^4 = F, R^5 = F)
57 (R^1 = H, R^2 = OMe, R^3 = H, R^4 = Cl, R^5 = Cl)

58 (R^1 = Me, R^2 = OMe, R^3 = H, R^4 = Cl, R^5 = Cl)

59 (R^1, R^2, R^3, R^4, R^5 = H)
60 (R^1, R^2, R^3, R^4 = H, R^5 = OMe)
61 (R^1, R^2, R^3 = H, R^4 = OMe, R^5 = H)
62 (R^1, R^2 = H, R^3 = OMe, R^4, R^5 = H)
63 (R^1, R^2, R^3 = H, R^4 = CN, R^5 = H)
64 (R^1, R^2, R^3, R^4 = H, R^5 = F)
65 (R^1, R^2, R^3 = H, R^4 = Cl, R^5 = H)
66 (R^1, R^2, R^3 = H, R^4 = F, R^5 = F)
67 (R^1, R^2, R^3 = H, R^4 = Cl, R^5 = Cl)

68 (R^1 = H, R^2 = OMe, R^3 = H, R^4 = F, R^5 = F)
69 (R^1 = H, R^2 = OMe, R^3 = H, R^4 = Cl, R^5 = Cl)

70 (R^1 = Me, R^2 = OMe, R^3 = H, R^4 = Cl, R^5 = Cl)

[a] Reagents and conditions: (a) Et_3N, $HgCl_2$/DMF, rt, overnight; (b) i. CF_3COOH, CH_2Cl_2, rt, overnight; ii. 2 M ethanolic HCl, EtOH (except for compounds **61** and **65**).

3. Results and Discussion

3.1. PBP1b Inhibitory Potencies

The synthesized compounds were first evaluated for their relative inhibition of the TG reaction of PBP1b from *E. coli* using a fluorescence assay.[18] The compounds that showed notable relative inhibitions at 500 μM were further characterized by determination of their IC_{50} values (Tables 1 and 2). The exact protocols are described in the Experimental Section. To allow direct comparisons of new compounds with the initial hit compound, we included the resynthesized hit in all of the assays performed. We used the hydrochloride salt of the hit compound (compound **46**, Table 1) and confirmed that there were no differences in the TG inhibition between these two forms of the compound (*see* Table 1 and Figure 1 for IC_{50} values determined for *E. coli* PBP1b).

Table 1. PBP1b inhibitory potencies of tryptamine-based derivatives **29-46**.

Compound	RA (%) of PBP1b (*E. coli*)[a] IC_{50} (μM)
29	93
30	80 ±13
31	86
32	96 ±35
33	94
34	97
35	54 ±16 **IC_{50} > 250 μM**
36	62 ±10 **IC_{50} > 250 μM**
37	15 ±4 **IC_{50} = 103 ±23 μM**
38	105 ±27
39	88
40	24 ±4 **IC_{50} = 193 ±7 μM**
41	75 ±29
42	118 ±28
43	28 ±8 **IC_{50} > 250 μM**
44	5 ±2 **IC_{50} = 104 ±2 μM**

45	31 ±12 **IC_{50} > 250 μM**
46 (hit compound)	1 ±1 **IC_{50} = 21 ±4 μM**

[a] RA, relative activity determined at 500 μM for each compound

Table 2. PBP1b inhibitory potencies of compounds with the guanidine moiety **59-70**.

Compound	**RA (%) of PBP1b (*E. coli*)[a]** **IC_{50} (μM)**
59	73 ±30
60	67 ±21
61	55 ±15 **IC_{50} > 250 μM**
62	29 ±5 **IC_{50} > 250 μM**
63	79 ±26
64	42 ±9 **IC_{50} > 250 μM**
65	15 ±2 **IC_{50} = 166 ±39 μM**
66	10 ±1.5 **IC_{50} = 97 ±39 μM**
67	1 ±2 **IC_{50} = 51 ±18 μM**
68	28 ±3 **IC_{50} > 250 μM**

69	9 ±5 **IC_{50} = 50 ±17 μM**
70	4 ±3 **IC_{50} = 39 ±9 μM**

[a] RA, relative activity determined at 500 μM for each compound

The results presented in Tables 1 and 2 allowed us to postulate the structural requirements for inhibition of TG activity of *E. coli* PBP1b. First, the results obtained with compounds **29**-**45** (Table 1) show that all of the modifications of the substituents on the tryptamine core led to slightly diminished inhibitory potencies in comparison with the hit compound **46**. For example, compound **44** (IC_{50} = 104 ±2 μM) showed an approximately 5-fold reduction in inhibition when compared to **46** (IC_{50} = 21 ±4 μM), despite only a minor structural change (SMe at position 5 on the indole ring substituted with an OMe group). In line with this result, the closest analog to compound **44** without the Me moiety at position 2 on the indole (compound **40**) showed a further decrease in inhibitory potency (IC_{50} = 193 ±4 μM).

However, when the amine moiety was transformed into guanidine (compounds **59**-**70**), a general increase in inhibitory potency was observed, regardless of the indole ring substitutions at positions 2 and 5. For instance, the guanidine-bearing compounds **65**-**67** (Table 2) were more potent than their amine counterparts **35**-**37** (Table 1). Similarly, in the 5-methoxytryptamine-substituted and the di-substituted group of compounds, the guanidines **69** (IC_{50} = 50 ±17 μM, Table 2) and **70** (IC_{50} = 39 ±9 μM, Table 2) outperformed the amine-bearing analogs **40** (IC_{50} = 193 ±7 μM, Table 1) and **44** (IC_{50} = 104 ±2 μM, Table 1).

The results also show that halogens on the benzyl ring attached on the indole nitrogen are necessary, as these compounds showed more pronounced inhibition. For example, the

unsubstituted benzyl derivatives (e.g. **29**, **41**, **45**) showed no inhibition, whereas their halogen-substituted counterparts (**37**, **44**, **46**) showed inhibition, with IC_{50} values in the micromolar range (Table 1). Chloro atoms attached to the benzyl moiety contributed more to the TG inhibition when compared with fluoro-substituted derivatives. This was clearly seen when the chlorobenzyl-substituted compounds **44** (IC_{50} = 104 ±2 μM) and **69** (IC_{50} = 50 ±17 μM) are compared with fluorobenzyl-substituted tryptamines **43** and **68**, which were both inactive (Tables 1 and 2). In addition, two halogens were more beneficial than the mono-halogen substitution pattern (e.g., compounds **35**, **65** *versus* **37**, **67**, and **34**, **64** *versus* **36**, **66**; Tables 1 and 2). All of the other substituents on the benzyl ring used in our SAR study (i.e., OMe, CN) led to diminished inhibition of TG activity, regardless of their position on the benzyl ring (compounds **30**-**33** and **60**-**63**; Tables 1 and 2).

Interestingly, no general SAR rules can be defined for the role of substituents at positions 2 (H or Me) and 5 (H, OMe, or SMe) on the core indole. For example, the influence of di-substitution was in some cases pronounced, as the removal of both moieties led to an approximately 5-fold reduced inhibition, as observed with the unsubstituted compound **37** (IC_{50} = 103 ±23 μM; Table 1) and the 2-methyl-5-methylthio-di-substituted **46** (IC_{50} = 21 ±4 μM; Table 1), whereas in some other cases no significant effects were observed (compare unsubstituted and di-substituted compounds **67** and **70**, which inhibited TG activity of PBP1b with IC_{50} values of 51 ±18 μM and 39 ±9 μM, respectively; Table 2).

3.2. Interactions with Lipid II

The *in-vitro* TG activity inhibition assay used measures the overall TG rate, and this can identify inhibitors that directly inhibit enzyme activity, as well as those that interfere with

substrate availability. As we have previously shown that binding to lipid II is an important mechanism of action of the initial hit compound, we decided to determine whether the compounds used in this study also bind lipid II.

To test for these specific interactions between selected compounds (**66**, **67**, **69**, **70**) and lipid II, a competition assay was set up with nisin, which uses lipid II for its pore-forming activity.[19,20] Adding nisin to carboxyfluorescein (CF)-loaded vesicles that contain lipid II results in leakage of the fluorescent dye from the vesicles (due to lipid II-dependent pore formation by nisin). We assayed inhibition of nisin activity through addition of 20 nM nisin to 20 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles containing 0.1 mol% lipid II, which gave about 55% leakage after 1 min. The inhibition by these compounds was determined in triplicate at 3 μM, and for compounds **69** and **70** also at 1.5 μM (stock solution, 1 mM in DMSO). The compounds were added to the cuvette with the vesicles 2 min before addition of nisin. The data in Figure 3 were determined 50 s after addition of nisin.

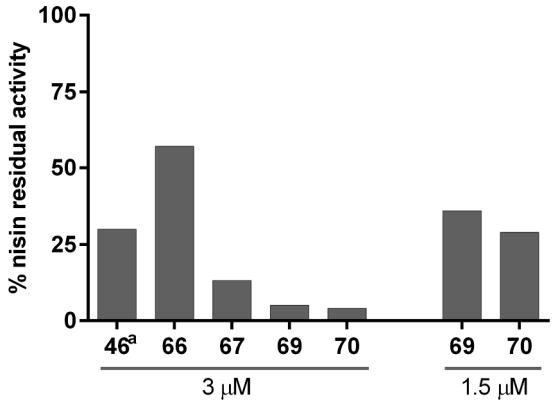


Figure 3. Interactions of the selected compounds with lipid II. The assays were performed at 3 μM compounds, and also 1.5 μM for compounds **69** and **70**. The inhibition was determined in triplicate, and the errors did not exceed 5%. [a]Hit compound.

In the presence of 3 μM compound **70**, more than 90% inhibition of nisin activity was achieved (i.e., significant reduction in the leakage of CF induced by nisin). These results suggest that all of these compounds bind to lipid II, and thus prevent its interaction with nisin. Among these compounds, **69** and **70** showed the most pronounced lipid II binding, which was significant also at 1.5 μM.

It was previously established that the amine residue of compound **46** (hit compound) was crucial in the interaction with the pyrophosphate group of lipid II.[15] Based on the results from Figure 3, it appeared that the guanidine moieties of these compounds can interact with the pyrophosphate group of lipid II to an even greater extent than the amine group of compound **46**. This is most probably due to more bonds (i.e., H-bonds) formed between the phosphate groups of lipid II and the basic guanidine moiety. This type of inhibition is reminiscent of several lipid II-targeting antibiotics, such as lantibiotic nisin, which binds the pyrophosphate of lipid II,[12] as well as vancomycin and related second-generation glycopeptides.[21] Moreover, bacitracin also inhibits peptidoglycan biosynthesis via binding to the pyrophosphate moiety of the lipid carrier.[22]

3.3. Antimicrobial Activities

For the compounds that showed enzyme inhibition, their *in-vitro* antibacterial activities were determined using a panel of four different bacterial strains: *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Enterococcus faecium*, and *Escherichia coli* (Table 3).

Table 3. Susceptibility of the panel of four bacterial strains to these tryptamine-based compounds.

Compound	MIC (μg/mL)[a]			
	S. aureus ATCC 25923	***E. coli*** ATCC 8739	***MRSA*** ATCC 43300	***E. faecium*** ATCC 19434
35	32 (100)	32 (100)	32 (100)	32 (100)
36	64 (198)	64 (198)	128 (397)	64 (198)
37	16 (45)	16 (45)	16 (45)	4 (11)
40	32 (83)	64 (166)	32 (83)	32 (83)
43	64 (174)	64 (174)	64 (174)	64 (174)
44	8 (20)	8 (20)	16 (40)	8 (20)
45	32 (92)	128 (369)	64 (184)	32 (92)
46 (hit compound)	16[b] (38)	256 (616)	4 (9.6)	32 (76)
61	32 (73)	32 (73)	64 (146)	128 (293)
62	32 (89)	> 128 (> 356)	32 (89)	16 (44)
64	32 (92)	64 (184)	128 (369)	64 (184)
65	64 (145)	64 (145)	64 (145)	32 (73)
66	16 (44)	16 (44)	32 (88)	32 (88)
67	8 (20)	16 (40)	8 (20)	4 (10)
68	32 (81)	32 (81)	64 (162)	128 (324)
69	8 (19)	16 (37)	8 (19)	8 (19)
70	4 (9.1)	32 (72)	4 (9.1)	4 (9.1)

[a] In the parentheses, the MIC values are expressed in μM.

[b] The free amine form of **46** (hit compound) had a MIC of 4 μg/mL against the *S. aureus* ATCC 25923 strain, as determined previously.[15] For compound **46**, the MIC for the same strain was 16 μg/mL.

Compounds **44**, **66**, **67**, **69**, and **70** showed the most promising antibacterial activities, with MICs from 4-32 μg/mL (Table 3). The best-performing compound in this series was compound **70**, as it showed antibacterial activities with MICs of 4 μg/mL (approx. 9 μM) against *S. aureus*, MRSA, and *E. faecium*. These results represent a clear (4- to 8-fold) improvement when compared with the starting compound **46** (initial hit compound). Unfortunately, all of these compounds (including **69** and **70**; Table 3) were less active against Gram-negative *E. coli*, which suggested poor permeation through the outer membrane. Very similar effects were also observed for some other indole-containing molecules in pre-clinical development.[23] Nevertheless, this reduction in activity for compounds **69** and **70** was generally less pronounced than for the parent compound **46**, which showed a 16-fold increase in the MIC when compared with, for example, *S. aureus* (Table 3).

3.4. Cytotoxicity Studies

To rapidly determine the possibility of non-specific cytotoxic effects of novel tryptamine-based compounds, we used a HEK 293 cell-viability assay. Here, only compounds that showed either *in-vitro* inhibition of TG activity (Tables 1 and 2) or antibacterial activity (Table 3) were used (Figure 4). In this cell-viability assay, compound **46** (hit compound) showed severe cytotoxicity against the HEK293 cells (Figure 4, Table S1). This is in agreement with the previously established data that compound **46** acts as a non-specific membrane disruptor.[15]

Interestingly, from a comparison of the cell viability data for compounds **44** and **46**, we can conclude that substitution of SMe with the OMe group at position 5 does not reduce the cytotoxicity, as both compounds had severe cytotoxic effects at 8 μg/mL (Figure 4). Similarly, omitting either only the Me group at position 2 (compound **40**) or both substituents at positions 2

and 5 on the indole ring (compound **37**) did not reduce the cytotoxicity, as **37** and **40** were still both severely cytotoxic towards HEK293 cells (Figure 4, Table S1). However, incorporation of the guanidine moiety instead of the amine almost completely eliminated the cytotoxicity of these compounds against HEK293 cells, as observed from the comparisons of the cell viabilities with the amine-bearing compounds **37**, **40**, **44** and the guanidine-based analogs **67**, **69**, **70** (Figure 4, Table S1). Moreover, replacement of the two chloro atoms (compound **67**) with fluoro atoms (compound **66**) also led to slightly reduced cytotoxic effects at 8 μg/mL (Figure 4). In line with previous data, omitting the Me group at position 2 on the indole ring only has a minor effect on cytotoxicity, as compounds **69** and **70** showed very similar effects.

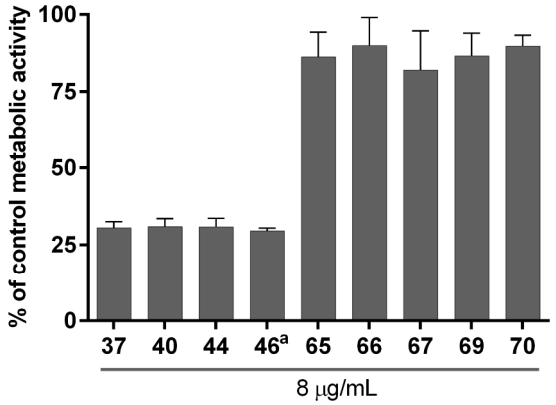


Figure 4. Cytotoxicity of compounds on HEK293 cells after 24 h treatment. The effects on cell viability of the compounds at 8 μg/mL were evaluated using the MTS assay. [a]Hit compound.

The cell viability assays shown in Figure 4 indicate that we can modify the starting compound **46** in such a way that its cytotoxicity is significantly reduced. Compound **70** showed the most promising characteristics in the other assays (IC_{50} in the low micromolar range, greatest affinity

for lipid II, good antimicrobial activity), and here it also showed very low cytotoxicity towards the HEK293 cells.

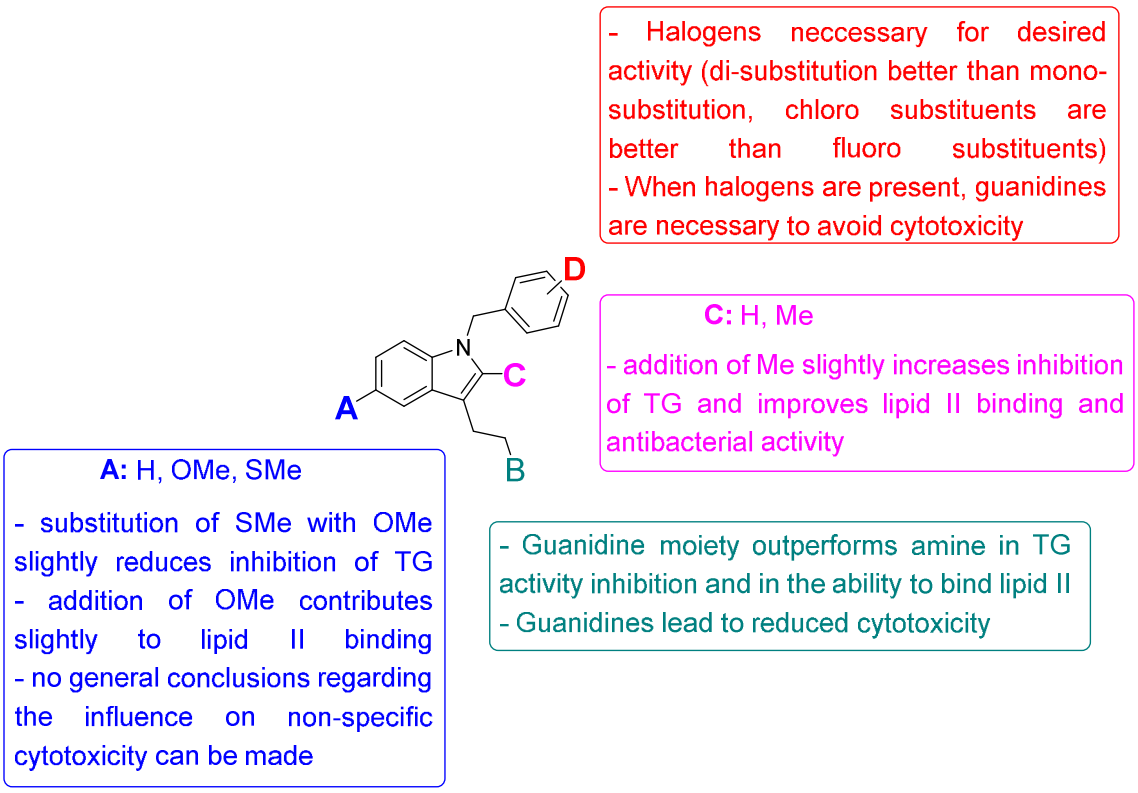


Figure 5. Schematic representation of the structural changes and their influence on *in-vitro* inhibition of TG activity, lipid II binding, antibacterial activity, and toxicity towards HEK293 cells.

4. Conclusions

We synthesized a focused library of tryptamine-based derivatives with the aim to explore the SARs for effective inhibition of TG activity (Figure 5). As the initial hit compound had several

drawbacks, the main goal was to reduce the unwanted characteristics of this compound, and concurrently to retain the desired inhibition of the TG reaction and the antibacterial activity.

Incorporation of structural changes into the core indole structure led to compounds with significantly better properties, in comparison with the hit compound. The most promising compounds from this series are compounds **69** and **70**, which represent significantly improved hit compounds for further development of inhibitors of TG activity. Both **69** and **70** are more drug-like and simpler compared to the known natural products that either target the TG active site or sequester the substrate lipid II. From the data presented herein, it appears that we are on the right path to achieve an appropriate balance between substituents that contribute to better inhibitory potencies and concurrently to lower non-specific cytotoxicities. Therefore, we believe that our results represent the basis for additional medicinal chemistry efforts towards more potent compounds that will show improved inhibition of the TG reaction, increased degree of specificity, and even better antibacterial activity. As the chemistry of indole-based derivatives is well known and highly versatile, variations at positions 3, 6, and 7 should provide additional chemical space exploration and further optimization.

5. Experimental Section

5.1. General Chemistry Methods

The reagents and solvents were obtained from commercial sources (Fluka, Sigma-Aldrich, Acros Organics, Alfa Aesar, TCI, Fluorochem). DOPC was purchased from Avanti Polar Lipids Inc. Nisin A was produced, isolated, and purified as described previously.[24] Lipid II and dansyl-lipid II were synthesized and purified as described elsewhere.[25] The solvents for the chemical

reactions were distilled before use, while the other chemicals were used as received. Analytical TLC was performed on Merck silica gel ($60F_{254}$) pre-coated plates (0.25 mm), with the compounds visualized under UV light and/or stained with the relevant reagent. Flash column chromatography was performed on Merck silica gel 60 (mesh 70-230), using the indicated solvents. Yields refer to the purified products, and they were not optimized. All of the melting points were determined on a Reichert hot-stage apparatus, and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance III NMR spectrometer and on a Bruker DPX Avance 400 MHz instrument equipped with a QNP probe. Measurements were made at a temperature of 295 K, and are reported in ppm using tetramethylsilane or solvent as an internal standard (DMSO-d_6 at 2.50 ppm, $CDCl_3$ at 7.26 ppm). The coupling constants (*J*) are in Hz, and the splitting patterns are designated as: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; td, triple doublet; t, triplet; dt, double triplet; and m, multiplet. ^{13}C NMR spectra were recorded on a Bruker Avance 400 DPX spectrometer at 302 K, and are reported in ppm using solvent as internal standard (DMSO–d_6 at 39.5 ppm). Mass spectra data and high-resolution mass measurements were performed on a VG-Analytical Autospec Q mass spectrometer. Infrared spectra were obtained on a Thermo Nicolet FT-IR spectrometer using the attenuated total reflection technique. Analytical purity of the assayed compounds was ≥95% unless stated otherwise, as determined by HPLC analyses. HPLC analyses were run on an Agilent 1100 system equipped with a quaternary pump and a multiple wavelength detector. An Agilent Eclipse C18 column was used (4.6 mm× 50 mm, 5 mm), with a flow rate of 1.0 mL/min, detection at 254 nm, and an eluent system of: A, H_2O with 0.1% TFA; B, MeOH. Different gradients were applied:

Gradient 1: 0-3 min, 40% B; 3-18 min, 40% B → 80% B; 18-23 min, 80% B; 23-30 min, 80% B → 40% B; run time, 30 min; temperature, 25 °C.

Gradient 2: 0-15 min, 20% B → 80% B; 13-16 min 80% B → 90% B; 16-19 min, 90% B; 19-20 min, 90% B → 10% B; temperature, 25 °C.

5.2. Protein Expression and Purification

The model protein used in this study (*E. coli* PBP1b) was produced and purified as previously described.[26]

5.3. *In-vitro* TG Activity and TG Inhibition

TG activity was monitored using the continuous fluorescence assay method.[18] For simultaneous screening of numerous conditions, this technique was adapted to a 96-well plate format (Greiner Bio-One). The standard reaction was carried out at 30 °C in 50 µL 50 mM Hepes, pH 7.5, 200 mM NaCl, 0.2% decyl-PEG, 10 mM $CaCl_2$, 20% DMSO, 10 µM dansyl-lipid II (prepared as described previously[19]), 1 U muramidase (Cellosyl) and 100 nM *E. coli* PBP1b. The data were collected for 30 min using a Victor 3 fluorimeter (Perkin Elmer) with excitation at 355 nm and emission at 536 nm.

The IC_{50} values of the compounds were determined using the same fluorescence-based assay. The initial rate of the reaction was determined in the presence of various concentrations of inhibitor (20-1500 µM) and plotted *versus* inhibitor concentration using the SigmaPlot program (Systas Software). The IC_{50} value is the inhibitor concentration that decreases the initial rate by a factor of 2.

5.4. MIC Determination

Tests were carried out using microtitration plates and 200 µL (final volume) Mueller-Hinton broth, following the European Committee on Antimicrobial Susceptibility Testing/ Clinical and Laboratory Standard Institute recommended procedure. The compounds were dissolved in 100% DMSO at 10 mg/mL, and 20-fold diluted in Mueller-Hinton broth just before use. The inocula were prepared for each strain by suspending isolated colonies from overnight cultured plates. Equivalents of 0.5 Mac Farland turbidity standard (approximately 1.10^8 CFU/mL) were prepared in saline solution (NaCl, 0.085%) and then diluted 100-fold in Mueller-Hinton broth. The MICs were determined as the lowest dilution of the product that showed no visual turbidity.

5.5. Preparation of Large Unilamellar Vesicles

Large unilamellar vesicles (LUVs) containing DOPC and 0.1 mol% lipid II were prepared essentially as described previously.[27] Dried lipid films were hydrated by addition of 50 mM CF (pH 7.5), followed by vigorous stirring, and these were then submitted to 10 freeze–thaw cycles. The LUVs were prepared by repeated extrusion through polycarbonate filters with a 200-nm pore size (Isopore membrane filters, Millipore, Ireland).[28] Following extrusion, the vesicles were passed through a Sephadex G-50 spin column (equilibrated with 10 mM Tris, 150 mM NaCl, pH 7.5) to remove the nonenclosed probe. The final phospholipid concentration was determined by phosphate analysis, according to Rouse et al.[29]

5.6. Carboxyfluorescein Leakage Assay in Large Unilamellar Vesicles

The lipid II binding affinities of the different compounds were determined by measuring their effects on lipid II-dependent pore-formation of the lantibiotic nisin. Any compound binding to lipid II resulted in reduced induction of CF leakage in response to nisin (20 nM). The compounds

were preincubated with CF-loaded, lipid II-containing vesicles (20 μM) for 2 min. None of the tested compounds induced CF leakage during this preincubation period. Then nisin was added, and the nisin-induced and lipid-II-dependent leakage was followed over time (emission wavelength 516 nm, excitation wavelength 492 nm). Finally, treatment with Triton X-100 induced total CF release, which was used to calculate the percentage of CF-leakage for that particular experiment. The potency of the compounds to inhibit the nisin induced leakage (hence bind to Lipid II) was expressed as % of residual nisin activity. This was calculated by determining the amount of leakage after 50 s in the presence of compound relative to the amount of leakage obtained in the absence of compound (determined in a separate control experiment) which was set to 100%.

5.7. Cell Culture

HEK293 cells were obtained from ATCC (LGC Standards, UK) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained in a humidified chamber at 37 °C and 5% CO_2.

5.8. Metabolic activity assay

The HEK293 cells were seeded at 2×10^4 cells/well in 100 μL medium in 96-well plates. Stock solutions of compounds in DMSO were prepared at 8 mg/mL and mixed with fresh medium, to achieve the final concentration of 8 μg/mL. The cells were allowed to adhere for 24 h. Then, the medium was removed and replaced with medium containing the compound of interest. The

control cultures received medium containing the appropriate vehicle. Treatments were performed in triplicate. The metabolic activities were assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer instructions. For IC_{50} determinations, various concentrations of the compounds of interest were used, in triplicate, in 96-well plates.

5.9. Chemical Syntheses

5.9.1. Procedure for preparation of 2,5-disubstituted tryptamines 5 and 6: Grandberg synthesis.

A suspension of 4-methoxyphenylhydrazine hydrochloride (**3**) (1.74 g, 10 mmol) or 4-thiomethylphenylhydrazine hydrochloride (**4**) (1.90 g, 10 mmol) in water (50 mL) was stirred for 10 min, followed by drop-wise addition of NaOH (2 M, 5 mL). During the addition, the corresponding phenylhydrazine began to precipitate. Immediately after completion of addition, the precipitate was filtered off and used in the next step.

To a solution of 4-methoxy- or 4-thiomethylphenylhydrazine (1 equiv.) in EtOH (60 mL) at 35 °C, 5-chloro-2-pentanone (85%, 1.4 equiv.) was slowly added, and the temperature of the reaction mixture was maintained between 35 °C and 40 °C. After the addition, the mixture was stirred for additional 24 h at 85 °C. The dark reaction mixture was then allowed to cool to room temperature, and the volatiles were removed *in vacuo*, yielding a dark brown oil. Water (100 mL) was added to the residue, followed by extraction with CH_2Cl_2 (5× 100 mL). The combined organic layers were dried with Na_2SO_4 and filtered, and the solvent was evaporated under

reduced pressure to yield a viscous dark oil. The products **5** and **6** were used in the next step without purification and spectroscopic characterization.

5.9.2. General procedure for synthesis of N-phthaloyl-protected tryptamines 7-10.

To a solution of a desired tryptamine derivative **1, 2**, **5**, or **6** (1 equiv.) in toluene (100 mL), phthalic anhydride (1.1 equiv.) and Et_3N (1.1 equiv.) were added. The reaction mixture was heated at reflux for 24 h with continuous removal of water with a Dean-Stark trap. After the reaction was complete (as monitored by TLC), the mixture was cooled to room temperature and washed consecutively with NaOH (1 M, 2× 50 mL), water (1× 50 mL), and HCl (1 M, 2× 50 mL). The organic phase was dried with Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure, to yield a viscous dark orange residue. The compounds **7**-**10** were subsequently purified by column chromatography or crystallization.

5.9.3. General procedures for preparation of benzylated derivatives 11-28.

Procedure A (for compounds **11**-**22**). To a suspension of NaH (60% wt. in mineral oil, 1.5 equiv.) in dry DMF (5 mL), a solution of the desired *N*-phthaloyl-protected tryptamine **7** or **8** (1 equiv.) in dry DMF (5 mL) was added. After stirring the reaction mixture at room temperature for 30 min, an appropriate benzyl bromide derivative (1.5 equiv.) was added. The reaction mixture was heated to 55 °C and stirred overnight under an argon atmosphere, followed by solvent evaporation under reduced pressure. The residue was dissolved in EtOAc (40 mL) and extracted consecutively with water (3× 30 mL), saturated aqueous solution of $NaHCO_3$ (1× 40 mL), and brine (1× 40 mL). The organic phase was dried with Na_2SO_4 and filtered, and

concentrated to give the crude products **11**-**22**, which were purified by column chromatography or crystallization.

Procedure B (for compounds **23**-**28**). To a stirred solution of the desired *N*-phthaloyl-protected tryptamine **9** or **10** (1 equiv.) in dry CH_3CN (20 mL), Cs_2CO_3 (3 equiv.) was added at room temperature. After 10 min of stirring, an appropriate benzyl bromide derivative (1.2 equiv.) was slowly added, and then the reaction mixture was stirred overnight at 80 °C under an argon atmosphere. After cooling the reaction mixture, the solvent was evaporated and the crude residue was partitioned between CH_2Cl_2 (50 mL) and water (50 mL). The organic phase was additionally washed with brine (2× 50 mL), dried with Na_2SO_4, filtered, and concentrated, to give crude products **23**-**28** that were purified by column chromatography.

5.9.4. General procedure for removal of the phthaloyl protective group: preparation of compounds 29-46.

To a suspension of a corresponding *N*-phthaloyl-protected compound **11**-**28** (1 equiv.) in EtOH (10 mL), hydrazine hydrate (5 equiv.) was added drop-wise at room temperature. The reaction mixture was then heated to 90 °C and the resulting solution stirred overnight under reflux. After the reaction was complete (as monitored by TLC), the mixture was cooled to room temperature and the white precipitate filtered off. The filtrate was concentrated under reduced pressure, diluted with aqueous NaOH (1 M, 5 mL), and extracted with EtOAc (3× 20 mL). The combined organic layers were dried with Na_2SO_4 and filtered, and the solvent was evaporated under reduced pressure. The products were subsequently purified by column chromatography using CH_2Cl_2/MeOH, 9/1 (saturated with gaseous NH_3) as eluent. The purified compounds were converted into hydrochloride salts **29**-**46** by dissolving the oily residues in absolute EtOH (5 mL)

and treating the solutions with an excess of freshly prepared ethanolic HCl (2 M). After evaporation of EtOH, the residues were triturated with Et_2O (4 mL) and the solids formed were filtered off to yield pure compounds **29-46**.

5.9.5. General procedure for synthesis of bis-Boc-protected guanidines 47-58.

To a solution of the desired tryptamine (unsubstituted derivatives **29-37**, 5-methoxytryptamines **39-40**, and 2-methyl-5-methoxytryptamine **44**) in DMF (5 mL), *N*,*N*'-di-(*tert*-butoxycabonyl)-*S*-methylisothiourea (1 equiv.), Et_3N (2 equiv.), and $HgCl_2$ (1 equiv.) were added consecutively at room temperature. The reaction mixture was stirred at room temperature overnight. After the reaction was complete (as monitored by TLC), the salt Hg(SMe)Cl was filtered off, and the filtrate evaporated under reduced pressure. To the residue, Et_2O (20 mL) was added, and the organic phase was extracted with water (3× 10 mL), saturated aqueous solution of $NaHCO_3$ (3× 10 mL) and brine (1× 10 mL). The organic layer was dried with Na_2SO_4, filtered, and concentrated, to give crude products **47-58**, which were purified by column chromatography.

5.9.6. General procedure for synthesis of final compounds 59-70.

The desired bis-Boc-protected compound **47-58** (1 equiv.) was dissolved in dry CH_2Cl_2, followed by addition of CF_3COOH (97%, 10 equiv.). The reaction mixture was stirred overnight under an argon atmosphere, and then the volatiles were evaporated under reduced pressure, to yield pure compounds that were converted into hydrochloride salts (**59-60**, **62-64**, **66-70**), except for compounds **61** and **65**. The hydrochlorides were obtained by first dissolving the residues in absolute EtOH (5 mL) and then treating the solutions with an excess of freshly prepared

ethanolic HCl (2 M). After evaporation of EtOH, the residues were triturated with Et_2O (4 mL), and the solids formed were filtered off, to yield pure compounds in their hydrochloride forms.

ASSOCIATED CONTENT

Supporting Information Available: Spectroscopic and analytical data for the intermediates and final compounds associated with the manuscript. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through the contributions of all of the authors. All of the authors have approved the final version of the manuscript.

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ABBREVIATIONS

CF, carboxyfluorescein; DMF, dimethylformamide; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; HEK, human embryonic kidney; LUV, large unilamellar vesicle; PBP, penicillin-binding protein; TP, transpeptidase; TG, transglycosylase.

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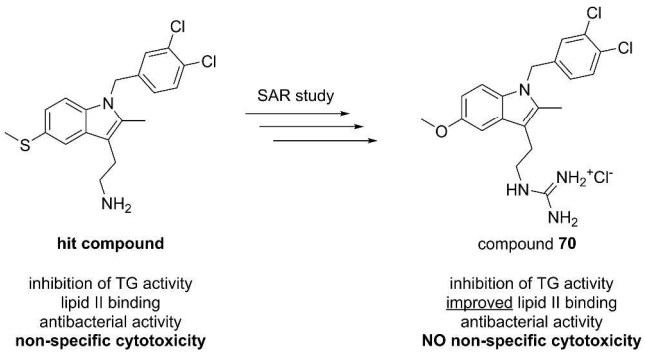
Table of Contents Graphic

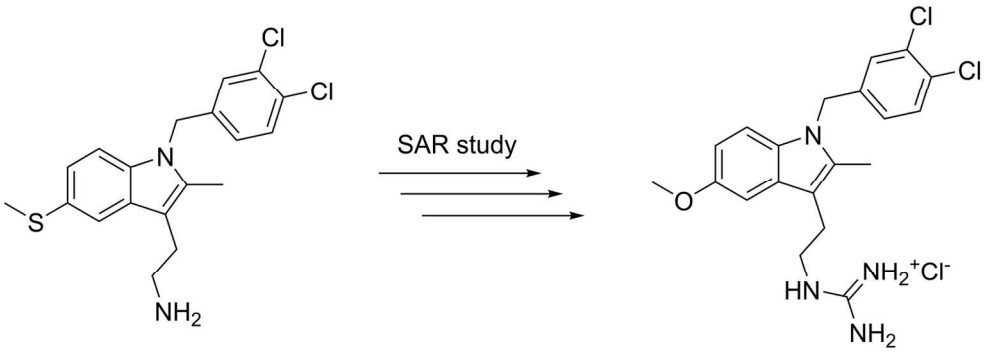


hit compound

inhibition of TG activity
lipid II binding
antibacterial activity
non-specific cytotoxicity

compound **70**

inhibition of TG activity
improved lipid II binding
antibacterial activity
NO non-specific cytotoxicity



hit compound

inhibition of TG activity
lipid II binding
antibacterial activity
non-specific cytotoxicity

compound **70**

inhibition of TG activity
improved lipid II binding
antibacterial activity
NO non-specific cytotoxicity

76x41mm (600 x 600 DPI)