Expedited Articles

Substituted Salicylanilides as Inhibitors of Two-Component Regulatory Systems in Bacteria

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A new class of inhibitors of the two-component regulatory systems (TCS) of bacteria was discovered based on the salicylanilide screening hits, closantel (**1**) and tetrachlorosalicylanilide (**9**). A systematic SAR study versus a model TCS, KinA/Spo0F, demonstrated the importance of electron-attracting substituents in the salicyloyl ring and hydrophobic groups in the anilide moiety for optimal activity. In addition, derivatives **8** and **16**, containing the 2,3-dihydroxybenzanilide structural motif, were potent inhibitors of the autophosphorylation of the KinA kinase, with IC_{50}s of 2.8 and 6.3 μM, respectively. Compound **8** also inhibited the TCS mediating vancomycin resistance (VanS/VanR) in a genetically engineered *Enterococcus faecalis* cell line at concentrations subinhibitory for growth. Closantel (**1**), tetrachlorosalicylanilide (**9**), and several related derivatives (**2**, **7**, **10**, **11**, **20**) had antibacterial activity against the drug-resistant organisms, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF).

Introduction

The spread of antibiotic resistance among pathogenic bacteria is increasingly compromising the clinical management of infectious diseases. The situation is especially acute in hospitals, where changes in the epidemiology and susceptibility patterns of Gram-positive cocci have led to a dramatic upsurge in multi-drug-resistant infections.[1] The 1990s have witnessed a significant increase in the prevalence of methicillin-resistant coagulase negative staphylococci (MRCNS) and methicillin-resistant *Staphylococcus aureus* (MRSA) due to new trends in patient care, an increase in the immunocompromised patient population, and the frequent misuse of antibacterial agents in clinical practice.[2] As a consequence, clinicians have become reliant on vancomycin as the sole broadly effective therapy, which, in turn, has led to the selection of newer vancomycin-resistant nosocomial pathogens. In particular, the incidence of enterococci resistant to vancomycin (particularly *Enterococcus faecium*, VREF) in US hospitals increased 20-fold during the period 1989 to 1993,[3] with as many as 20% of enterococcal isolates exhibiting some level of vancomycin resistance.[4] Mortality rates as high as 60% have been reported for patients with VREF bacteremia,[5] for which antiinfective therapy is extremely limited.

Clearly there is a need for antibacterial agents with a novel mechanism of action.[6] Recent reports from Japan[7] and the United States[8] of the isolation of an intermediate vancomycin-resistant strain of *S. aureus* (VISA) have only heightened this sense of urgency.

The two-component regulatory systems (TCS) of bacteria[9−11] are logical targets for chemotherapeutic intervention because homologous proteins have been identified only in yeast and fungi[12] and not in higher eukaryotes. These signal transduction systems are intimately involved in the maintenance of bacterial cell homeostasis and the expression of virulence determinants, including high level vancomycin resistance in enterococci, in response to external and internal environmental stimuli. In simplest terms, the TCS are composed of an environmental sensing protein (the histidine protein kinase) and a DNA-binding protein (the response regulator). The activation or repression of gene transcription is achieved through the transfer of phosphate from ATP to a specific aspartic acid residue of the response regulator via a high-energy phosphohistidine intermediate of the cognate kinase. This stands in sharp contrast to eukaryotic signal transduction systems which employ ATP-dependent serine/threonine and tyrosine protein kinases, and consequently, there exists a strategic opportunity for the design of selective antibiotics.

Proteins within the histidine protein kinase and response regulator families share considerable sequence homology, especially in regions involved in the autophosphorylation and phosphotransfer functions. Furthermore, over 100 TCS have been identified to date in such important nosocomial pathogens as *S. aureus*, *E. faecium*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Therefore, a single inhibitor may be expected to interact

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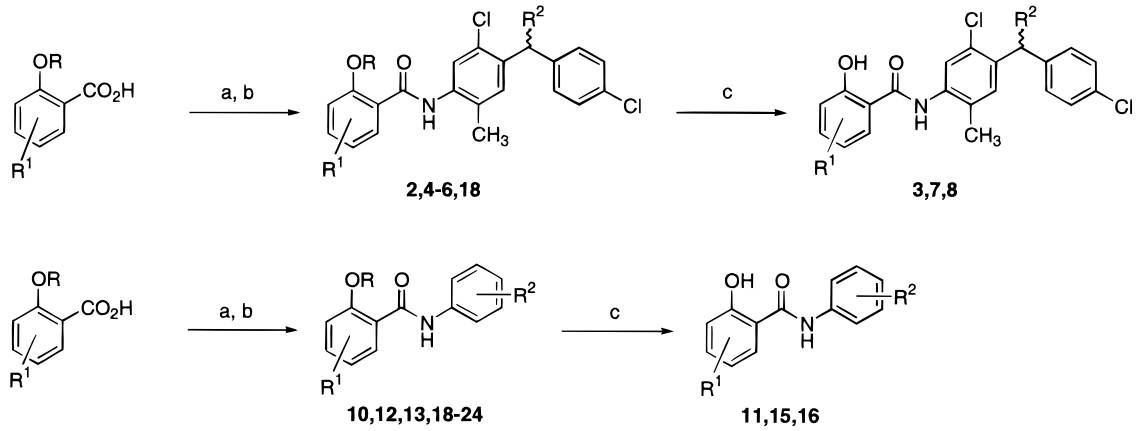
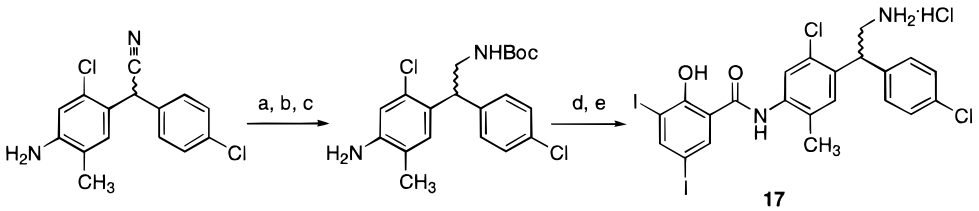
Scheme 1[a]



[a] Reagents: (a) R = H: $SOCl_2$, C_6H_6, 60 °C; R = CH_3: $(COCl)_2$, DMF (cat.), THF; (b) R = H: substituted aniline, dioxane, 50 °C; R = CH_3: substituted aniline, THF; (c) R = CH_3: BBr_3, CH_2Cl_2 or pyridine hydrochloride, 190 °C.

Scheme 2[a]



[a] Reagents: (a) H_2/Ra-Ni, AcOH, Ac_2O; (b) concentrated HCl, EtOH, heat; (c) $(Boc)_2O$, NEt_3; (d) 3,5-diiodosalicyloyl chloride, dioxane, 50 °C; (e) TFA, anisole, CH_2Cl_2, $NaHCO_3$; (f) HCl, IPA.

with several TCS within a single bacterium and diminish the potential for chromosomal resistance emergence.

Inhibitors of TCS controlling the expression of specific virulence factors potentially could be used as adjunct therapy when combined with traditional bactericidal agents. Furthermore, agents that inhibit multiple TCS simultaneously or that inhibit an essential TCS may be effective as sole therapy by virtue of their global effect on bacterial cell metabolism. In this regard, TCS essential for bacterial cell growth have been identified in *Caulobacter crescentus*, where they play a key role in the regulation of DNA replication and the cell cycle.[13,14]

Screening of our chemical library against KinA/Spo0F,[15] a model TCS which regulates sporulation in *Bacillus subtilis*, revealed that the veterinary anthelmintic agent, closantel (**1**),[16] and the topical antibacterial, 3,3′,4′,5-tetrachlorosalicylanilide (**9**),[17] inhibited autophosphorylation of the KinA kinase. Furthermore, both compounds exhibited potent antibacterial activity against resistant Gram-positive pathogens, including MRSA and VREF. In light of the structural similarity of these screening hits, we embarked on a focused SAR study to define the molecular basis for their TCS inhibitory effects.

Chemistry

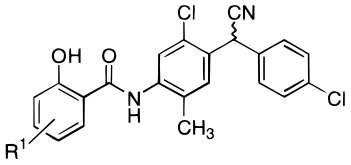
Salicylanilides were synthesized by acylation of the appropriate aniline with a substituted salicyloyl chloride in dioxane[16] (Scheme 1). Occasionally, it was preferable to use a substituted anisoyl chloride because of its synthetic accessibility or commercial availability. In such cases, it was necessary to demethylate the aromatic ether with pyridinium hydrochloride or boron tribromide in methylene chloride. For the synthesis of **17**, the required aniline was prepared from 4-chlorophenyl-(2-chloro-4-amino-5-methylphenyl)cyanomethane by reduction of the nitrile and protection of the resulting amine as the Boc derivative. Acylation with 3,5-diiodosalicyloyl chloride followed by deprotection under standard conditions afforded the target compound (Scheme 2).

Closantel (**1**) was prepared by the reported procedure.[16] Compound **18** is mentioned in the literature;[18] however, no physical or spectroscopic data are reported. Although salicylanilides **10**, **19**, **20**, and **22**−**24** are known,[17,19] the compounds were preferentially synthesized as in Scheme 1. The 3,5-dimethoxysalicylic acid[20] for the synthesis of **4** and **12**, the 2-methoxy-5-trifluoromethylbenzoic acid for the synthesis of **7** and **15**,[21] and the 3,5-bis(trifluoromethyl)salicylic acid[22] for the synthesis of **3** and **11** were prepared by literature procedures. Compounds **9** and **14** were commercially available.

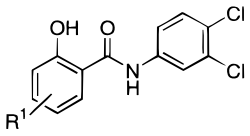
Results and Discussion

Salicylanilides uncouple the energy-yielding from the energy-conserving reactions in mitochondria and other energy-transducing membranes by discharging the protonmotive force required for ATP synthesis.[23] In the presence of an uncoupling agent, mitochondrial preparations exhibit increased oxygen uptake without ADP, a phenomenon known as the release of state 4 respiration. The toxicity of the salicylanilides to both mammalian and bacterial cells has largely been ascribed to this general effect on energy metabolism. We were concerned, therefore, that these compounds could inhibit the TCS nonspecifically by a mechanism related to the

Table 1. Effect of Salicyloyl Ring Modification on Inhibition of KinA Autophosphorylation[a]



1 - 8

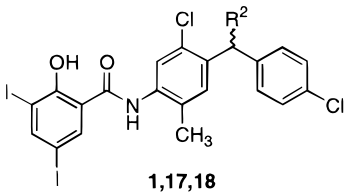


9 - 16

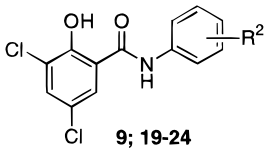
compd	R^1	IC_{50} (μM)[b]	compd	R^1	IC_{50} (μM)[b]
1	3,5-I_2	3.8	**9**	3,5-Cl_2	45
2	3,5-Cl_2	4.8	**10**	3,5-I_2	21
3	3,5-$(CF_3)_2$	9.0	**11**	3,5-$(CF_3)_2$	27
4	3,5-$(OCH_3)_2$	250	**12**	3,5-$(OCH_3)_2$	>500
5	3,5-(*t*-Bu)$_2$	>500	**13**	3,5-(*t*-Bu)$_2$	>500
6	5-Cl	>500	**14**	5-Cl	>500
7	5-CF_3	19	**15**	5-CF_3	>500
8	3-OH	2.8	**16**	3-OH	9.5/3.0[c]

[a] The biochemical assay was run as described in ref 24 with minor modifications. [b] IC_{50} values are the mean of two experimental determinations with interassay variability <50%. [c] Results of each experiment reported due to >50% variability.

Table 2. Effect of Anilide Ring Modification on Inhibition of KinA Autophosphorylation[a]



1,17,18



9; 19-24

compd	R^2	IC_{50} (μM)[b]
1	CN	3.8
17	CH_2NH_2	86
18	=O	>500
9	3,4-Cl_2	45
19	4-Cl	185
20	4-Cl; 3-CF_3	50
21	4-*t*-Bu	67
22	H	>500
23	4-NO_2	>500
24	4-OCH_3	>500

[a] The biochemical assay was run as described in ref 24 with minor modifications. [b] IC_{50} values are the mean of two experimental determinations with interassay variability <50%.

effects on uncoupling oxidative phosphorylation. Accordingly, our initial modifications to the core structure of the salicylanilides were made to the salicyloyl ring to ensure that both the closantel (**1**−**8**) and the tetrachlorosalicylanilide (**9**−**16**) subseries exhibited parallel structure−activity relationships versus KinA/Spo0F. Direct comparison of analogues revealed that compounds **1**−**8**, bearing the more hydrophobic anilide moiety found in closantel (**1**), were consistently more potent in the biochemical assay than the corresponding analogues in the tetrachlorosalicylanilide series, **9**−**16** (Table 1). Nevertheless, substituent effects were comparable in both subseries, suggesting that these compounds inhibit KinA autophosphorylation by a similar mechanism and at the same site. In particular, optimal activity was achieved when two electron-withdrawing groups were present on the salicyloyl ring (see compounds **1**−**3** and **9**−**11**). Replacement of the halogens with electron-donating (compounds **4** and **12**) or bulky, hydrophobic (compounds **5** and **13**) substitutents dramatically reduced or completely abolished activity. Removal of one of the substituents from the 3-position of the salicyloyl ring, as in derivatives **6**, **14**, and **15**, adversely affected activity. The sole exception was compound **7**, containing the 5-trifluoromethyl substituent, which retained significant inhibitory potency. Inhibition of KinA autophosphorylation could also be achieved through introduction of a 3-hydroxy group, catechols **8** and **16** being the most potent inhibitors in the present series.

In a similar fashion, systematic modification of the anilide moieties of closantel and tetrachlorosalicylanilide was carried out to further define the structure−activity relationships as TCS inhibitors (Table 2). Comparison of the potencies of derivatives **17** and **18** with closantel (**1**) demonstrated that activity was highly dependent on the nature of the substituent linking the phenyl rings in this subseries. In particular, the greater than 20-fold decrease in potency for the aminomethyl derivative **17** may be due to the introduction of a charged functionality in a portion of the molecule which binds to a hydrophobic region of the kinase. The inactivity of the 4-benzoyl derivative **18** may be a reflection of the conformational rigidity imposed by the sp^2-hybridized keto group.

In the tetrachlorosalicylanilide subseries, potency was dependent on a delicate balance of electronic and hydrophobic effects. Whereas the 4-chloro analogue **19** was less potent than the parent compound **9**, analogues **9**, **20**, and **21** exhibited comparable activities in the enzyme assay, despite the fact that the hydrophobic anilide substituents vary in their electronic and steric properties. In contrast, derivatives without substituents (**22**) or containing more polar functionalities (**23** and **24**) are essentially devoid of activity.

Several of the salicylanilides were tested for their in vitro effects on growth of Gram-positive bacterial pathogens, including MRSA and VREF (Table 3). Generally, the compounds exhibited consistent levels of antibacterial activity against both susceptible and resistant organisms compared to the clinically useful antibiotics, oxacillin and vancomycin. Interpretation of these bacterial susceptibility results in terms of a global effect on TCS cell signaling, however, is dangerous in light of the recognized effect of salicylanilides on energy metabolism. Therefore, it is likely these agents are affecting multiple targets in the whole cell and that antibacterial activity may be the product of inhibition of several vital biochemical processes.

The activities of compounds **8**, **16**, **22**, and **23** reported here lend further support to this hypothesis. The catechol analogues **8** and **16** are potent TCS inhibitors (IC_{50}s versus KinA/Spo0F = 2.8 and 6.3 μM, respectively). On the basis of the known structure-uncoupling relationships of salicylanilides,[23] however, these compounds would not be expected to demonstrate significant uncoupling activity. As shown in Table 3, these agents exhibit modest antibacterial activity (MIC's = 8−32 μg/mL). Conversely, compounds **22** and **23** have been reported to be potent releasers of state 4 respiration in mitochondria[19] but are inactive in the TCS biochemical assay. As with derivatives **8** and **16**, derivatives **22** and **23** exhibit only moderate antibacterial activity against *S. aureus* (MIC's = 1−4 μg/mL) and little or no activity

Table 3. Minimum Inhibitory Concentrations (MIC's) and VanS/VanR Activity for Selected Salicylanilides

	MIC's (μg/mL)[a]				VanHLuc[b]	
compd	*S. aureus* ATCC29213	MRSA OC2089	*E. faecalis* OC3041	*E. faecium* OC3312[c]	activity	AG_{80}[d] (%)
1	2	1	0.12	1	−	100
2	1	1	2	2	+ {i}[e]	
7	1	1	1	1	−	95
8	16	16	16	16	+	66
9	0.5	0.25	0.5	1	+ {i}[e]	
10	1	0.25	0.5	0.5	+ {i}[e]	
11	1	1	2	2	+ {i}[e]	
16	8	8	8	32	+[f]	48
20	0.5	0.25	0.5	1	− {i}[e]	
21	2	2	8	4	− {i}[e]	
22	1	4	64	32	NT[g]	
23	4	4	>32	>64	NT[g]	
vancomycin	1	2	4	>128	NT[g]	
oxacillin	0.25	>64	16	>64	NT[g]	

[a] Determination of susceptibility was performed using the broth microdilution method of the National Committee for Clinical Laboratory Standards (see ref 25). The variance in the determination of MIC values is 2-fold such that MIC differences of ≥2 dilutions are significant. [b] The activity of the VanS/VanR TCS is measured through changes in the expression of a protein fusion between VanH (a D-specific α-ketoacid dehydrogenase involved in vancomycin resistance) and firefly luciferase (a gene expression reporter) in *Enterococcus faecalis* OC3364, containing *vanR vanS* and the Φ(*vanH-luc*) gene fusion on different plasmids (see the Experimental Section). The interaction of a compound with the VanS/VanR TCS may lead to one of two possible outcomes: (1) a decrease of the luciferase specific activity relative to an untreated control (+) or (2) induced overexpression of luciferase specific activity (+ {i}). Positive compounds affect expression of luciferase (1) significantly more than growth or (2) at concentrations subinhibitory for growth. To identify compounds causing overexpression of luciferase through alternate mechanisms, inducers were also tested against *E. faecalis* OC3365, containing Φ(*vanH-luc*), but lacking *vanR vanS*. Compounds that were inducers and positive caused overexpression of luciferase in OC3364, but not in OC3365; whereas compounds that were inducers and negative (− {i}), caused overexpression of luciferase in both strains. [c] Vancomycin-resistant strain. [d] Percentage of specific activity relative to untreated control at a concentration allowing 80% growth. [e] Inducer. [f] Based exclusively on AG_{80}; indistinct effects on luciferase expression at subinhibitory concentrations. [g] Not tested.

against enterococci (MIC's 32 to >64 μg/mL). Studies are currently underway to better define the mechanism of action of these compounds at the cellular level.

All of the salicylanilides exhibited minimal activity versus *E. coli* in standard broth microdilution assays. However, levels of antibacterial activity comparable to those manifested against Gram-positive organisms were observed in a mutant strain of *E. coli* containing a leaky outer membrane and a wild-type *E. coli* treated with polymyxin-B nonapeptide, a membrane-permeabilizing agent. These results suggest that Gram-negative bacteria also contain the target(s) of inhibitory action of the salicylanilides, but that the outer membrane of this class of bacteria is a formidable barrier to penetration of the compounds.

To provide a clearer measure of the ability of these salicylanilides to affect TCS signaling in the intact bacterial cell, select compounds were tested in a whole-cell reporter-gene assay (Table 3). The test system consists of an *Enterococcus faecalis* cell line which expresses the VanS/VanR TCS mediating vancomycin resistance and VanH-luc, a fusion protein comprised of an α-ketoacid dehydrogenase involved in vancomycin resistance (VanH) and firefly luciferase (luc). Interaction of a test compound with the VanS/VanR TCS is detected through a change in the luciferase specific activity relative to an untreated control. Several compounds (**2**, **8**, **9**, **10**, **11**, and **16**) affected the VanS/VanR-dependent expression of VanH-luc at concentrations subinhibitory for growth (Table 3). Because the effects observed on the VanS/VanR-regulated gene occurred at concentrations of compound that did not affect growth, the effect must be specific and cannot be a consequence of an overall inhibition of protein synthesis.

A more detailed summary of the data for compound **8** is depicted in Figure 1. This catechol derivative clearly inhibited both bacterial cell growth (Figure 1A) and TCS-dependent expression of the reporter gene (Figure 1B) in a concentration-dependent fashion. The effects on growth became significant, however, only at concentrations ≥2 μM, whereas a specific effect on luciferase expression could be detected at concentrations as low as 0.5 μM. This result suggests that compounds such as **8** specifically inhibit TCS in the living cell distinct from a general effect on protein synthesis. Furthermore, the inhibition of VanS/VanR provides proof of principle for the design of agents which target TCS-mediated resistance mechanisms in pathogenic bacteria. In contrast, a few of the compounds (**1**, **7**, **20**, and **21**) either had no specific effect on VanS/VanR or affected the levels of expression of the fusion protein through mechanisms not involving the two-component regulatory system (see footnotes in Table 3). In these cases, the test compound may disturb energy metabolism at concentrations below the apparent affinity for the VanS histidine protein kinase.

Conclusion

We have identified a series of salicylanilide inhibitors of bacterial two-component regulatory systems based on the screening hits closantel (**1**) and tetrachlorosalicylanilide (**9**). Potency versus the KinA/Spo0F model system is primarily dependent on the electron-withdrawing properties of the substituents on the salicyloyl ring and the hydrophobicity of the anilide moiety. Although the antibacterial activity of these salicylanilides presumably results from multiple mechanisms working in concert, data from a reporter-gene assay in a genetically engineered *E. faecalis* cell line show that inhibition of TCS regulating bacterial cell homeostasis and adaptive responses occurs at concentrations that have little effect on the bacterial growth rate. Although unlikely to be effective chemotherapeutic agents themselves due to their effect on mitochondrial respiration,

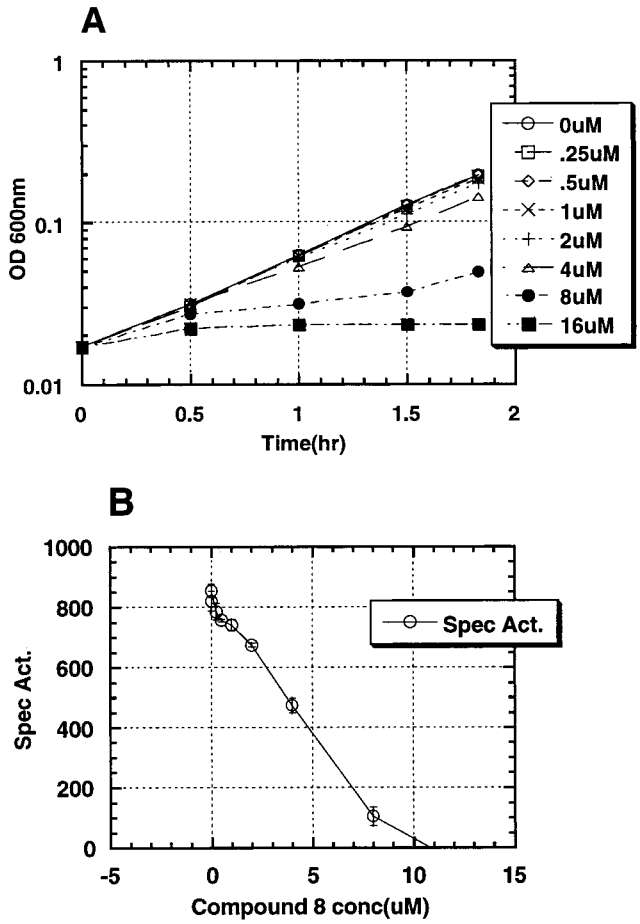


Figure 1. Panel A: Effect of increasing concentrations of compound **8** on growth of *Enterococcus faecalis* OC3364 compared to control growth (0 μM) in the presence of a subinhibitory concentration of vancomycin. Panel B: Effect of compound **8** on the VanS/VanR-mediated expression of luciferase specific activity (RLU/OD_{600}), measured 2 h after induction by vancomycin.

these salicylanilides may provide a structural template for the design of the next generation of selective TCS inhibitors. In particular, compounds that have minimal antibacterial activity but inhibit VanS/VanR in the whole cell, like **8**, **16**, or structurally related analogues, may be effective adjunct therapy for the treatment of vancomycin-resistant enterococcal infections.

Experimental Section

Melting points were determined on a Thomas-Hoover Meltemp apparatus and are uncorrected. The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 300 MHz on a Bruker AC-300 spectrometer using tetramethylsilane (δ 0.00) as an internal standard. Infrared (IR) spectra, obtained as KBr pellets, and combustion analyses were performed by Quantitative Technologies Inc., Whitehouse, NJ. Mass spectra were obtained on a Hewlett-Packard 5989A quadrupole mass spectrometer. Silica gel (E. Merck, 230−400 mesh) was used for all flash chromatography. Thin-layer chromatography was performed on Analtech silica gel GF prescored plates (250 μm).

General Procedure for Preparation of Salicylanilides 2, 4−6, 10, 12, 13, and 18−24. *N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-5-chloro-2-hydroxybenzamide (6). To a suspension of 5-chlorosalicylic acid (173 mg, 1.0 mmol) in dry benzene (10 mL) was added thionyl chloride (594 mg, 5.0 mmol) and catalytic DMF (2 drops) with stirring at room temperature. The resulting solution was heated at 60 °C for 1 h, and then the solvent was removed in vacuo to afford a gummy yellow solid. The 5-chlorosalicyloyl chloride so isolated was used without purification in the next step. To a solution of 4-chlorophenyl-(2-chloro-4-amino-5-methylphenyl)cyanomethane (152 mg, 0.52 mmol) in 1,4-dioxane (5 mL) was added 5-chlorosalicyloyl chloride (100 mg, 0.52 mmol), and the reaction mixture was heated at 50 °C. After 1 h the reaction was quenched with 2 N NaOH (5 mL) and the aqueous mixture washed with ethyl acetate. The aqueous layer was separated, neutralized with 1 N HCl, and extracted with ethyl acetate (20 mL). The organic extract was washed with brine (20 mL), dried over $MgSO_4$, and concentrated in vacuo. The crude solid was recrystallized from chloroform/hexane to afford **6** (85 mg, 38%) as white crystals: mp 144−146 °C; ^{1}H NMR ($CDCl_3$) δ 11.52 (s, 1H), 8.04 (s, 1H), 7.76 (s, 1H), 7.51−7.26 (m, 6H), 7.02 (d, J = 8.7 Hz, 1H), 5.59 (s, 1H), 2.36 (s, 3H). Anal. ($C_{22}H_{15}Cl_3N_2O_2 \cdot 0.5H_2O$) C, H, N.

***N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-3,5-dichloro-2-hydroxybenzamide (2):** yield 70%; mp 209−210 °C; ^{1}H NMR ($CDCl_3$) δ 12.34 (br s, 1H), 10.18 (s, 1H), 8.03 (d, J = 2.3 Hz, 1H), 7.53 (d, J = 2.3 Hz, 1H), 7.38−7.31 (m, 6H), 5.59 (s, 1H), 2.31 (s, 3H); IR 2257, 1591 cm^{-1}; MS (CI) m/z 481 (M + H). Anal. ($C_{22}H_{14}Cl_4N_2O_2$) C, H, N.

***N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2-hydroxy-3,5-dimethoxybenzamide (4):** yield 78%; mp 192−194 °C; ^{1}H NMR ($CDCl_3$) δ 9.60 (s, 1H), 8.57 (s, 1H), 7.33−7.16 (m, 7H), 6.69 (d, J = 2.9 Hz, 1H), 5.60 (s, 1H), 3.95 (s, 3H), 3.85 (s, 3H), 2.34 (s, 3H); MS (CI) m/z 472 (M + H). Anal. ($C_{24}H_{20}Cl_2N_2O_4 \cdot 0.5H_2O$) C, H, N.

***N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-3,5-di-*tert*-butyl-2-hydroxybenzamide (5):** yield 67%; mp 92−95 °C; ^{1}H NMR ($CDCl_3$) δ 12.13 (s, 1H), 8.09 (s, 1H), 7.80 (s, 1H), 7.55 (s, 1H), 7.37−7.29 (m, 6H), 2.34 (s, 3H), 1.44 (s, 9H), 1.34 (s, 9H); MS (CI) m/z 524 (M + H). Anal. ($C_{30}H_{32}Cl_2N_2O_2$) C, H, N.

***N*-(3,4-Dichlorophenyl)-2-hydroxy-3,5-diiodobenzamide (10):** yield 78%; mp 192−194 °C (lit.[17] mp 198−199 °C); ^{1}H NMR ($CDCl_3$) δ 13.17 (s, 1H), 10.18 (s, 1H), 8.31 (d, J = 1.9 Hz, 1H), 8.16 (d, J = 1.9 Hz, 1H), 7.90 (d, J = 2.4 Hz, 1H), 7.66 (dd, J = 2.3, 8.8 Hz, 1H), 7.24 (d, J = 8.8 Hz, 1H); IR 1532 cm^{-1}; MS (CI) m/z 534 (M + H).

***N*-(3,4-Dichlorophenyl)-2-hydroxy-3,5-dimethoxybenzamide (12):** yield 93%; mp 173−175 °C; ^{1}H NMR ($CDCl_3$) δ 9.47 (br s, 1H), 7.94 (s, 1H), 7.61 (br s, 1H), 7.50−7.42 (m, 2H), 7.10 (d, J = 2.8 Hz, 1H), 6.67 (d, J = 2.8 Hz, 1H), 3.94 (s, 3H), 3.83 (s, 3H); IR 1590 cm^{-1}; MS (CI) m/z 343 (M + H). Anal. ($C_{15}H_{13}Cl_2NO_4$) C: calcd, 52.65; found, 54.29; H; N: calcd, 4.09; found, 3.17.

***N*-(3,4-Dichlorophenyl)-3,5-di-*tert*-butyl-2-hydroxybenzamide (13):** yield 47%; mp 161−163 °C; ^{1}H NMR ($CDCl_3$) δ 12.12 (s, 1H), 7.84 (s, 1H), 7.80 (s, 1H), 7.54−7.45 (m, 2H), 7.26 (s, 1H), 7.24 (d, J = 2.0 Hz, 1H), 1.57 (s, 9H), 1.44 (s, 9H); IR 1582 cm^{-1}; MS (CI) m/z 395 (M + H). Anal. ($C_{21}H_{25}Cl_2NO_2$) C, H, N.

***N*-[5-Chloro-4-(4-chlorobenzoyl)-2-methylphenyl]-3,5-diiodo-2-hydroxybenzamide (18):** yield 32%; mp 247−248.5 °C; ^{1}H NMR (DMSO-d_6) δ 8.36 (s, 1H), 8.24 (s, 1H), 7.78 (s, 1H), 7.76 (d, J = 8.5 Hz, 2H), 7.67 (d, J = 8.5 Hz, 2H), 7.50 (s, 1H), 2.28 (s, 3H). Anal. ($C_{21}H_{13}Cl_2I_2NO_3$) C, H, N.

***N*-(4-Chlorophenyl)-3,5-dichloro-2-hydroxybenzamide (19):** yield 60%; mp 193−195 °C (lit.[19] mp 190−191 °C); ^{1}H NMR (DMSO-d_6) δ 12.53 (br s, 1H), 10.72 (br s, 1H), 8.08 (s, 1H), 7.83 (s, 1H), 7.73 (d, J = 8.6 Hz, 2H), 7.47 (d, J = 8.6 Hz, 1H). Anal. ($C_{13}H_8Cl_3NO_2$) C, H, N.

***N*-(4-Chloro-3-trifluoromethylphenyl)-3,5-dichloro-2-hydroxybenzamide (20):** yield 50%; mp 140−142 °C (lit.[17] mp 134−135 °C); ^{1}H NMR (DMSO-d_6) δ 12.15 (br s, 1H), 10.95 (br s, 1H), 8.26 (s, 1H), 8.03−8.00 (m, 2H), 7.84 (s, 1H), 7.77 (d, J = 8.8 Hz, 1H). Anal. ($C_{14}H_7Cl_3F_3NO_2$) C, H, N.

***N*-(4-*tert*-Butylphenyl)-3,5-dichloro-2-hydroxybenzamide (21):** yield 60%; mp 159−161 °C; ^{1}H NMR (DMSO-d_6) δ 12.97 (br s, 1H), 10.60 (br s, 1H), 8.16 (d, J = 2.3 Hz, 1H),

7.83 (d, J = 2.3 Hz, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 8.6 Hz, 2H), 1.29 (s, 9H). Anal. ($C_{17}H_{17}Cl_2NO_2$) C, H, N.

***N*-Phenyl-3,5-dichloro-2-hydroxybenzamide (22):** yield 45%; mp 135−136 °C (lit.[19] mp 124−125 °C); ^{1}H NMR (DMSO-d_6) δ 12.81 (br s, 1H), 10.65 (br s, 1H), 8.14 (d, J = 2.0 Hz, 1H), 7.83 (d, J = 2.0 Hz, 1H), 7.69 (d, J = 7.9 Hz, 2H), 7.41 (m, 2H), 7.20 (t, J = 7.2 Hz, 1H). Anal. ($C_{13}H_9Cl_2NO_2$) C, H, N.

***N*-(4-Nitrophenyl)-3,5-dichloro-2-hydroxybenzamide (23):** yield 30%; mp >250 °C (lit.[19] mp >330 °C); ^{1}H NMR (DMSO-d_6) δ 12.62 (br s, 1H), 10.74 (br s, 1H), 8.24 (d, J = 9.3 Hz, 2H), 8.15 (d, J = 2.2 Hz, 1H), 8.01 (d, J = 9.3 Hz, 2H), 7.56 (d, J = 2.2 Hz, 1H). Anal. ($C_{13}H_8Cl_2N_2O_4$) C, H, N.

***N*-(4-Methoxyphenyl)-3,5-dichloro-2-hydroxybenzamide (24):** yield 25%; mp 173−174 °C (lit.[19] mp 173−174 °C); ^{1}H NMR (DMSO-d_6) δ 13.09 (br s, 1H), 10.57 (br s, 1H), 8.15 (d, J = 2.4 Hz, 1H), 7.82 (d, J = 2.4 Hz, 1H), 7.58 (d, J = 8.6 Hz, 2H), 6.98 (d, J = 8.6 Hz, 2H). Anal. ($C_{14}H_{11}Cl_2NO_3$) C, H, N.

General Procedure for Preparation of Salicylanilides 3 and 11. *N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2-hydroxy-3,5-bis(trifluoromethyl)benzamide (3). To a suspension of 2-methoxy-3,5-bis(trifluoromethyl)benzoic acid (120 mg, 0.42 mmol) in THF (10 mL) were added oxalyl chloride (63 mg, 0.50 mmol) and catalytic DMF (0.2 mL) with stirring. The mixture was stirred at room temperature for 1 h. Pyridine (0.1 mL) and 4-chlorophenyl-(2-chloro-4-amino-5-methylphenyl)cyanomethane (150 mg, 0.52 mmol) were added, and the mixture was stirred at room temperature for an additional 2 h. The reaction mixture was quenched with 1 N HCl (10 mL) and extracted with ethyl acetate (20 mL). The organic extract was sequentially washed with concentrated HCl (10 mL), brine (10 mL), saturated sodium bicarbonate (10 mL), and brine (10 mL) and dried over $MgSO_4$. After evaporation of the solvent, {5-chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2-methoxy-3,5-bis(trifluoromethyl)benzamide was obtained (185 mg, 79%) as a yellow solid: mp 185−187 °C; ^{1}H NMR ($CDCl_3$) δ 8.58 (s, 1H), 8.53 (s, 1H), 8.07 (s, 1H), 7.37 (d, J = 9 Hz, 2H), 7.35 (s, 1H), 7.32 (d, J = 9 Hz, 2H), 5.61 (s, 1H), 4.03 (s, 3H), 2.37 (s, 3H); IR 3240, 1687, 1658 cm^{-1}; MS (CI) m/z 561 (M + H).

To a solution of {5-chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2-methoxy-3,5-bis(trifluoromethyl)benzamide in CH_2Cl_2 was added BBr_3 (1.0 M in CH_2Cl_2, 1 mL, 1 mmol) with stirring at room temperature. The reaction mixture was stirred for 3 h at room temperature and then evaporated to dryness. Water was added (20 mL) and the mixture extracted with ethyl acetate (20 mL). The organic extract was washed with brine (20 mL), dried over $MgSO_4$, and concentrated in vacuo. Flash chromatography on silica gel (hexane/ethyl acetate/acetic acid, 8:2:0.1) gave **3** (95 mg, 73%) as a white solid: mp 172−173 °C; ^{1}H NMR ($CDCl_3$) δ 12.05 (br s, 1H), 8.57 (s, 1H), 7.98 (s, 1H), 7.96 (s, 1H), 7.54 (s, 1H), 7.51 (d, J = 8 Hz, 2H), 7.41 (d, J = 8 Hz, 2H), 6.04 (s, 1H), 2.32 (s, 3H); IR 3345, 1656 cm^{-1}; MS (ES) m/z 547 (M + H). Anal. ($C_{24}H_{14}Cl_2F_6N_2O_2$) C, H, N.

***N*-(3,4-Dichlorophenyl)-2-hydroxy-3,5-bis(trifluoromethyl)benzamide (11):** yield 97%; mp 148−150 °C; ^{1}H NMR (DMSO-d_6) δ 11.55 (s, 1H), 8.63 (s, 1H), 8.05 (m, 2H), 7.62 (m, 2H); IR 3442, 1661, 1607 cm^{-1}; MS (ES) m/z 416 ($M^+ - 1$). Anal. ($C_{15}H_7Cl_2F_6NO_2$) C, H, N.

General Procedure for Preparation of Salicylanilides 7, 8, 15, and 16. *N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2-hydroxy-5-(trifluoromethyl)-benzamide (7). *N*-{5-chloro-4-[(4-chlorophenyl)cyanomethyl-2-methylphenyl}-2-methoxy-5-(trifluoromethyl)benzamide (prepared as described for **3** above) (816 mg, 1.65 mmol) and pyridine hydrochloride (16.2 g, 0.14 mol) were heated at 190 °C with stirring for 2 h under a nitrogen atmosphere. The melt was cooled and the solidified mass triturated with 3 N HCl. The precipitated solid was filtered, washed with 3 N HCl and water, and dried. Flash chromatography on silica gel (ethyl acetate/methylene chloride, 1:1) gave **7** (348 mg, 44%) as a white solid: mp 120 °C; ^{1}H NMR (DMSO-d_6) δ 11.57 (s, 1H), 8.28 (s, 1H), 7.81 (d, J = 8.3 Hz, 1H), 7.53−7.38 (m, 6H), 7.21 (d, J = 8.3 Hz, 1H), 6.04 (s, 1H), 2.36 (s, 3H); IR 3329−2930, 1649, 1619, 1586 cm^{-1}. Anal. ($C_{23}H_{15}Cl_2F_3N_2O_2$) C, H, N.

***N*-{5-Chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl}-2,3-dihydroxybenzamide (8):** yield 10%; mp 172−174 °C; ^{1}H NMR (DMSO-d_6) δ 11.29 (br s, 1H), 10.58 (s, 1H), 9.79 (br s, 1H), 8.21 (s, 1H), 7.52−7.38 (m, 6H), 7.01 (d, J = 7.6 Hz, 1H), 6.81 (t, J = 7.6 Hz, 1H), 6.03 (s, 1H). Anal. ($C_{22}H_{16}Cl_2N_2O_3 \cdot 0.1H_2O$) C, H, N.

***N*-(3,4-Dichlorophenyl)-2-hydroxy-5-(trifluoromethyl)-benzamide (15):** yield 46%; mp 184−185 °C; ^{1}H NMR (DMSO-d_6) δ 12.07 (br s, 1H), 10.67 (s, 1H), 8.12 (d, J = 2.1 Hz, 2H), 7.74 (d, J = 8.6 Hz, 1H), 7.70−7.63 (m, 2H), 7.18 (d, J = 8.6 Hz, 1H); IR 3113, 1636, 1618, 1586 cm^{-1}; MS (CI) m/z 350 (M + H). Anal. ($C_{14}H_8Cl_2F_3NO_2$) C, H, N.

***N*-(3,4-Dichlorophenyl)-2,3-dihydroxybenzamide (16):** yield 20%; mp 204−206 °C; ^{1}H NMR ($CDCl_3$) δ 11.72 (br s, 1H), 9.64 (br s, 1H), 7.97 (d, J = 2.2 Hz, 1H), 7.61 (dd, J = 8.7 Hz, 2.1 Hz, 1H), 7.43−7.40 (m, 2H), 7.11 (br s, 1H), 7.08 (d, J = 7.5 Hz, 1H), 6.81 (t, J = 7.5 Hz, 1H); MS (CI) m/z 299 (M + H).

***N*-{4-[2-Amino-1-(4-chlorophenyl)ethyl]-5-chloro-2-methylphenyl}-3,5-diiodo-2-hydroxybenzamide hydrochloride (17).** 4-Chlorophenyl-(2-chloro-4-amino-5-methylphenyl)cyanomethane (874 mg, 3.0 mmol) was hydrogenated at 50 psi for 21 h in a mixture containing Raney nickel (1.0 g), acetic acid (6 mL), and acetic anhydride (15 mL). After filtration through Celite, the reaction mixture was neutralized with saturated $NaHCO_3$ and extracted with ethyl acetate (3 × 100 mL). The combined ethyl acetate extracts were washed with brine (50 mL), dried over $MgSO_4$, and concentrated in vacuo to give a white solid. Recrystallization from ethyl acetate/hexane gave *N*-[2-(4-chlorophenyl)-2-(2-chloro-4-acetylamino-5-methylphenyl)]ethylacetamide (750 mg, 66%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 9.33 (br s, 1H), 7.97 (t, J = 4.3 Hz, 1H), 7.54 (s, 1H), 7.35 (s, 1H), 7.33 (d, J = 8.1 Hz, 2H), 7.23 (d, J = 8.1 Hz, 2H), 4.53 (t, J = 7.7 Hz, 1H), 3.76−3.51 (m, 2H), 2.21 (s, 3H), 2.07 (s, 3H), 1.74 (s, 3H).

N-[2-(4-Chlorophenyl)-2-(2-chloro-4-acetylamino-5-methylphenyl)]ethylacetamide (750 mg, 2.0 mmol) was dissolved in a mixture of ethanol (5 mL) and concentrated HCl (5 mL), and the mixture was heated to reflux temperature for 24 h. The reaction mixture was adjusted to pH 5 with 2 N NaOH and then extracted with ethyl acetate (3 × 100 mL). The combined extracts were dried over $MgSO_4$, and the solvent was removed in vacuo. The residue was recrystallized from ethyl acetate to give 2-(4-chlorophenyl)-2-(2-chloro-4-amino-5-methylphenyl)ethylamine hydrochloride (426 mg, 65%) as a white solid: ^{1}H NMR (DMSO-d_6) δ 8.03 (br s, 3H), 7.39 (d, J = 8.3 Hz, 2H), 7.31 (d, J = 8.3 Hz, 2H), 7.17 (s, 1H), 6.64 (s, 1H), 5.15 (br s, 2H), 4.56 (t, J = 7.7 Hz, 1H), 3.49−3.34 (m, 2H), 2.05 (s, 3H).

2-(4-Chlorophenyl)-2-(2-chloro-4-amino-5-methylphenyl)-ethylamine hydrochloride (264 mg, 0.80 mmol) was dissolved in THF (5 mL) containing Et_3N (122 μL, 0.90 mmol). The mixture was cooled to 5 °C and treated with di-*tert*-butyl dicarbonate (182 mg, 0.80 mmol). The reaction was stirred for 16 h and concentrated in vacuo. Flash chromatography on silica gel (hexane/ethyl acetate, 7:3) afforded *N*-*tert*-butoxycarbonyl-2-(4-chlorophenyl)-2-(2-chloro-4-amino-5-methylphenyl)ethylamine (300 mg, 95%): ^{1}H NMR ($CDCl_3$) δ 7.25 (d, J = 8.6 Hz, 2H), 7.17 (d, J = 8.6 Hz, 2H), 6.89 (s, 1H), 6.68 (s, 1H), 4.55−4.42 (m, 2H), 3.81−3.52 (m, 3H), 2.12 (s, 3H), 1.41 (s, 9H).

N-{4-[2-(*N*′-*tert*-Butoxycarbonylamino)-1-(4-chlorophenyl)-ethyl]-5-chloro-2-methylphenyl}-3,5-diiodo-2-hydroxybenzamide was prepared from *N*-*tert*-butoxycarbonyl-2-(4-chlorophenyl)-2-(2-chloro-4-amino-5-methylphenyl)ethylamine (300 mg, 0.76 mmol) and 3,5-diiodosalicylic acid (296 mg, 0.76 mmol) as described for **6** above.

The crude product was dissolved in CH_2Cl_2 (20 mL) and treated with trifluoroacetic acid (5 mL) containing a few drops of anisole. After 30 min the reaction mixture was neutralized

with saturated $NaHCO_3$ and extracted (3 × 50 mL) with ethyl acetate. The combined extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness in vacuo. The residue was dissolved in isopropyl alcohol (10 mL), and 1 equiv of HCl in isopropyl alcohol was added. *N*-{4-[2-Amino-1-(4-chlorophenyl)ethyl]-5-chloro-2-methylphenyl}-3,5-diiodo-2-hydroxybenzamide hydrochloride was collected by filtration (160 mg, 30%, two steps): ^{1}H NMR (DMSO-d_6) δ 13.22 (br s, 1H), 8.34 (s, 1H), 8.18 (s, 1H), 8.03 (br, 4H), 7.65 (s, 1H), 7.45 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 8.4 Hz, 2H), 4.76−4.69 (m, 1H), 3.63−3.49 (m, 2H), 2.29 (s, 3H). (Anal. ($C_{22}H_{18}Cl_2I_2N_2O_2$·HCl) C, H, N.

Autophosphorylation Assays. Inhibition of the autophosphorylation of two-component signal transduction system kinase was measured using the KinA/Spo0F regulatory system of *B. subtilis*.[15] The phosphorylation assay[24] was conducted in a 40 μL volume containing 100 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 20 μg/mL phenylmethanesulfonyl fluoride, and 10% glycerol. The proteins were added to the medium (final concentrations of KinA and Spo0F were 16.6 μg/mL and 22.3 μg/mL, respectively), and the reaction was initiated by the addition of 37.5 μCi of [γ-^{32}P]ATP and enough unlabeled ATP to give a final concentration of 0.1 mM. After 10 min incubation at room temperature the reaction was stopped by the addition of 10 μL of 5X loading buffer (250 mM Tris-HCl [pH 6.8], 10% glycerol, 0.02% bromophenol blue, 1%SDS, and 150 mM β-mercaptoethanol), and loaded onto a 10−20% gradient SDS-polyacrylamide gel, with a stacking gel of 5%. After electrophoresis, the gel was dried following removal of the lower portion to reduce background radioactivity. The dried gel was exposed in a Bio-Rad GS-250 Molecular Imager, and the radioactivity in the kinase band quantitated. Band densities were plotted versus inhibitor concentrations. IC_{50} values were determined from best-fit curves as concentrations of compound producing 50% inhibition.

VanH-luc Whole Cell Assay. The activity of the VanS/VanR TCS is measured through changes in the expression of a protein fusion between VanH (a D-specific α-ketoacid dehydrogenase involved in vancomycin resistance) and firefly luciferase (a gene expression reporter). The assay uses *Enterococcus faecalis* OC3364, derived from strain JH2, containing *vanR vanS* and the Φ(*vanH-luc*) gene fusion on different plasmids. Tested compounds and vancomycin (0.4 μg/mL final concentrated) were added to OC3364 cells growing exponentially in Todd Hewitt broth. At 30 min intervals samples were removed and growth was monitored by measuring optical density at 600 nm (OD_{600}). After 2 h of growth, cells were lysed by shaking for 10 min in an equal volume of Genepol X-80 mix. Luciferase activity was measured as Relative Light Units (RLU) in the presence of ATP, coenzyme A, and luciferin. Specific activity = RLU/OD_{600}.

MIC Determination. Determination of susceptibility was performed following the broth microdilution method of the National Committee for Clinical Laboratory Standards.[25] Bacteria are American Type Culture Collection (ATCC) strains and clinical strains and include a methicillin-resistant *S. aureus* and a vancomycin-resistant *E. faecium*.

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