Probe Report: C094-0010 – A Small Molecule That Targets a Signaling Pathway in AIP-Dependent Bacterial Quorum Sensing of *S. aureus* Agr3 agr locus genotype

Project Title: Small Molecule Inhibition of Staphylococcus Aureus Virulence
Grant Number: NIH **1 X01 MH078952-01**

Screening Center Name: New Mexico Molecular Libraries Screening Center
Principal Investigator of Screening Center: Larry A. Sklar, Ph.D., University of New Mexico

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PubChem Assay Identifiers (AID): 527;700;1014;1015, 1206 (Summary)

Assay or Pathway Target: AIP-Dependent Bacterial Quorum Sensing

Probe PubChem Compound Identifier (CID): 3240990 (N-2,3-dihydro-1,4-benzodioxin-6-yl-2-(3-methylpiperidin-1-yl)-2-phenylacetamide)

Mode of Action: The probe inhibits an element in the autoinducing pheromone (AIP) signaling pathway downstream of AIP binding to cellular receptors. The probe inhibits AIP induced virulence factor production in an *S. aureus* strain expressing the Agr3 variant of the accessory gene regulator (*agr*) locus, but has little or no effect on an Agr1 variant strain. Its inhibitory effects are thus selective for AIP signaling associated with the Agr3 genotype. The targeted pathway element(s) remain to be determined.

Overview: Quorum sensing is a cell-to-cell communication system that permits members of a bacterial population to coordinate their behavior dependent on cell density. The mediators of this communication system are small, diffusible pheromones or autoinducers that are secreted by the bacteria and that accumulate extracellularly. At the appropriate concentration threshold that reflects a sufficient number or quorum of bacteria, the autoinducers signal gene expression programs that direct the coordinated switch of the population to a virulence-associated phenotype. The synthesis of virulence factors by *S. aureus* is globally controlled by the accessory gene regulator (*agr*) locus, which encodes a two-component signaling pathway whose activating ligand is *agr*-encoded autoinducing pheromone (AIP). Several *agr* locus variants have been identified in *S. aureus* such that *agr* locus expression by one variant can inhibit *agr* locus expression by another and vice-versa. Thus, although similar virulence factors are produced in the end, cross-inhibition of gene expression by *agr* locus variants indicates heterogeneity in underlying signaling pathways. **C094-0010** is a novel inhibitor of the RNAIII promoter activation response induced in *S. aureus* by AIP. The IC_{50} is in the range of 100 – 200 nM and maximal effects (90% or greater inhibition) can be achieved at 12 μM concentrations in *in vitro* experimental systems. Under such conditions it also inhibits AIP-induced production of virulence factors α-hemolysin and lipase in Agr3 but not Agr1 strains of *S. aureus*. Receptor binding studies with FITC-AIP indicate that it does not significantly inhibit the binding of AIP to the AgrC cellular receptor, the initial step in the bacterial quorum sensing pathway. Cell viability is unaffected under these conditions for up to 3 hours at 37ºC. **C094-0010** is recommended as a probe of AIP-induced signaling

pathways in strains of *S. aureus* expressing the Agr3 variant of the *agr* locus. The specific pathway element(s) targeted by probe **C094-0010** remain to be determined.

Assay Provider Information

Specific Aims:

Quorum sensing is a cell-to-cell communication system that permits members of a bacterial population to coordinate their behavior dependent on cell density (for review see ref. #1). The mediators of this communication system are small, diffusible pheromones or autoinducers that are secreted by the bacteria and that accumulate extracellularly. At the appropriate concentration threshold that reflects a sufficient number or quorum of bacteria, the autoinducers signal gene expression programs that direct the coordinated action of the population. The list of bacterial pathogens that use this method of communication to regulate virulence is expanding and now includes some of the most common bacterial pathogens of humans including the medically important pathogen *Staphylococcus aureus*. (1). Because antibiotic resistance is an emerging problem in this pathogen (2) and vaccines are of limited efficacy (3), quorum sensing is becoming a therapeutic target for treatment of this infection (4-7). Attacking virulence by these strategies, termed "quorum quenching," has proven successful in two animal models of *S. aureus* infection (4, 7, and 8). We recently published data demonstrating that phagocyte-derived reactive oxidants inactivate the peptide thiolactone autoinducer of *S. aureus* and that this is important for host defense against this infection (8). Our data demonstrate that targeting virulence by chemical inactivation of the quorum sensing pheromone represents a viable treatment option. **We hypothesize that small molecule inhibitors of the peptide autoinducing pheromone (AIP) can abrogate virulence dependent gene expression.** To test this hypothesis, we are applying to the Molecular Libraries Screening Centers Network (MLSCN) to pursue the following specific aims:

Specific Aim #1: To screen libraries of small molecules in a high throughput fluorescence-based screening assay to identify compounds capable of suppressing pheromone-dependent activation of the promoter for a global regulator of *Staphylococcus aureus* virulence, RNAIII.

Specific Aim #2: To confirm that the compounds that inhibit RNAIII promoter activation also inhibit expression of the virulence genes that are regulated by RNAIII.

Significance:

Staphylococcus aureus permanently colonizes the moist squamous epithelium of the anterior nares of 20% of the population and is transiently associated with another 60%. Colonization is a known risk factor for invasive disease both in the hospital and the community and the resulting infections can range from superficial skin infections such as abscesses and impetigo to serious invasive infections such as septic arthritis, osteomyelitis, endocarditis, and toxic shock. Hospitalized patients who have been catheterized or who have undergone surgery are at increased risk of infection (9). Treatment of infections with antibiotics has become increasingly difficult owing to the widespread occurrence of strains that are antibiotic resistant including meticillin (formerly methicillin)-resistant *Staphylococcus aureus* (MRSA)(2, 10). Furthermore, the isolation of MRSA strains that are also resistant to vancomycin, the last drug for which the organism had been uniformly sensitive, raises the spectre of a return to the pre-antibiotic era. Moreover, treatment and care for patients with these infections results in significant costs to the health care system (11).

Of current concern is the emergence of community-acquired MRSA strains that are hypervirulent due in part to the expression of the Panton-Valentine Leukocidin (PVL) toxin that is associated with spontaneous skin and soft-tissue infections and necrotizing pneumonia (10, 12). Fatal community-acquired MRSA pneumonia and bacteremia was first reported in the United States among children in 1999 and in older adults with underlying diseases in 2005. However, it has now been described in immunocompetent young adults (10). Because vaccine trials have demonstrated limited efficacy for prevention of this infection and because this pathogen expresses virulence factors that subvert both innate and adaptive immunity (3, 9), there is considerable interest in developing new therapeutic agents for treatment of these more severe antibioticresistant infections. However, pharmaceutical companies have expressed minimal interest in attacking this problem (13) demonstrating the need for alternative mechanisms to screen compounds with antistaphylococcal activity. We believe that screening small molecule inhibitors of the genetic mechanism for upregulation of virulence factor production represents such a mechanism.

Rationale:

Targeting virulence by antagonism and/or inactivation of AIP. Structure-function studies of the *agr* peptide pheromones produced by *S. aureus* and the variations in the *agr*C receptor that binds these pheromones have determined the nature of the interactions and the sequences that are essential for activation and for inhibition of activation (6, 7, and 17-19). These studies have permitted synthesis of a universal peptide inhibitor (6) that inhibits RNAIII promoter activation both *in vitro* and *in vivo* (6, 7). In addition, our own work has demonstrated that oxidant modification of specific amino acids can inhibit binding and signaling through *agr*C (8). Together, these observations indicate that the receptor-ligand interaction that regulates quorum sensing dependent virulence is hydrophobic and that increases in polarity of the peptide can block binding. These data indicate the feasibility of inhibiting quorum sensing either by hydrophobic compound antagonism of peptide binding or by chemical modification of the peptide to prevent its binding.

Screening small molecule inhibitors of virulence for treatment of infectious disease. The feasibility of treating infectious diseases by inhibition of virulence (13) has been experimentally verified (20-23). Compounds were screened from various libraries to identify small molecule inhibitors that either altered virulence gene expression (20, 23) or inhibited toxin activity (21, 22). Moreover, these small molecule inhibitors enhanced host defense during *in vivo* infection (21-23). These publications provide “proof of principle” for this approach and suggest that therapeutic drugs for the treatment of infection can be identified by this mechanism.

Background:

Substantial progress has been made in understanding the molecular mechanisms for virulence regulation, including quorum sensing, in this pathogen. Quorum sensing in *S. aureus* is regulated in part by the accessory gene regulator (*agr*) operon (14-16). The *agr* locus combines secretion of an autoinducing peptide thiolactone (AIP) ligand and a two component regulatory pathway to generate a regulatory RNA transcript RNAIII that is the effector of the operon. All *S. aureus* strains can be categorized into four groups based on the amino acid sequence of the AIP produced (1, 17). Whereas clinical isolates are enriched for type I AIP, all four groups are represented in human disease (14-16). Under conditions of high autoinducer concentration, i.e. high bacterial density, RNAIII downregulates gene

expression encoding for surface adhesions while upregulating those encoding for capsule production, secreted toxins like PVL, proteases, and metabolic pathways (14, 15). The only protein product generated directly from RNAIII is delta hemolysin from the *hld* gene. This conversion from a tissue-adhering to a tissue-damaging and phagocyte-evading phenotype is thought to be important for in vivo pathogenesis and the development of invasive infection (4, 7, 8, and 14). *Agr* deletion mutants are less virulent in several animal models of infection including endocarditis, arthritis, pneumonia, and skin infection (14, 15). Direct demonstration of RNAIII production by bacteria in a lavage of a skin abscess (4, 8) indicates that quorum sensing is essential for acute infection at entry sites. Our recently published data suggest that phagocyte-derived reactive oxidants contribute to the control of in vivo quorum sensing in a skin infection model (8). Thus, the interaction of the pathogen with innate host defenses at the site of infection may dictate the type and course of the infection, i.e. acute and rapidly toxic vs. chronic and indolent. This raises the issue of how human infection occurs if the infecting strains secrete virulence pheromones that are inactivated by innate effectors. At least
two contributing factors that are not mutually exclusive could be involved: 1) These infections are opportunistic and occur primarily in hosts with defects in these innate effectors; and 2) The pathogen has mechanisms for producing virulence pheromones that can escape destruction by these effectors. Understanding all these aspects of the pathogenesis of staphylococcal infection, from the innate effectors that inhibit quorum sensing to the mechanisms that the pathogen uses to escape this control, are necessary to design drugs that target quorum sensing-dependent virulence.

Screening Center Information

Assay Implementation and Screening

PubChem Bioassay Name: Inhibitors of Bacterial Quorum Sensing

List of PubChem Bioassay Identifiers (AIDs): 527, 700, 1014, 1015

PubChem Primary Assay Description (AID 527):

A fluorescence based RNAIII promoter activation assay was developed using wild-type *agr* type 1 *S. aureus* that had been engineered with a plasmid encoding the promoter for RNAIII driving expression of GFP. When these reporter bacteria are in the early exponential phase of growth before quorum sensing has occurred, they are non-fluorescent. However, incubation with synthetic type 1 AIP induces GFP production and therefore fluorescence over the next 3 hours. Incubation of the reporter bacteria alone during this time frame is not sufficient to induce fluorescence indicating that minimal endogenous synthesis of AIP occurs under these conditions. The AIP activity is indicated by the intensity of GFP florescence.

Protocol. *Staphylococcus aureus* bacterial stocks SA RN6390: RNAIII-GFP (RNAIII), stored at -80 degrees C) are thawed and diluted with Columbia Broth (CB, Difco) to a final concentration of ~2.5x10^7 cfu/ml. AIP is added to a final concentration of 0.1 microM and the bacteria/AIP cultures are added to wells of a 384-well plate at 18 microL/well. To each well is then added 2 microL of test compound (1 milliM stock in DMSO pre-diluted 1:50 in phosphate-buffered saline (PBS)) to result in final concentrations of 2 microM test compound

and 0.2% (V/V) DMSO. Control wells are added with bacterial cultures containing AIP alone (AIP activation control or AAC), AIP plus the AIP inhibitor Erythrosin B (AIP inactivation control or AIC) and no additive (no activation control or NAC). Assay plates are then incubated 3 hours at 37 degrees C on a rocker. At the end of incubation, 40 microL of PBS containing 0.1% (V/V) Triton X-100 is added to each well. Well contents are mixed and plates subsequently stored on ice in the dark until analyzed by flow cytometry.

The HyperCyt high throughput flow cytometry platform (24,25) was used to sequentially sample cells from wells of 384-well microplates (2 microL/sample) for flow cytometer presentation at a rate of 40 samples/min. The resulting time-resolved data files were analyzed with IDLeQuery software (developed to support specialized analysis of time-resolved flow cytometry data files generated with the HyperCyt platform) to determine compound activity in each well.

The following data were obtained for each well: 1) the number of events analyzed, 2) the median fluorescence intensity of the bacteria, 3) the mean fluorescence intensity of the bacteria, 4) the % GFP negative bacteria, and 5) the % GFP positive bacteria. The number of events was used to assess the possibility of cytotoxicty mediated by the test compound, indicated by detection of less than 25% of the average number of events observed for all wells of a plate. The fluorescence intensity threshold for distinguishing %GFP positive bacteria was defined as the intensity level above which 90% of AAC control bacteria were observed.

Test compound inhibition of AIP activity was calculated by two methods. The first inhibition determination was on the basis of %GFP-positive cells (%I_GP) as follows:

%I_GP = 100 x [1 – (%Pos_test - %Pos_NAC/(%Pos_AAC - %Pos_NAC)]

in which %Pos_test, %Pos_NAC, and %Pos_AAC represented the %GFP positive cells in wells containing test compound, the average %GFP-positive cells in AIP no activation control (NAC) wells and the average %GFP-positive cells in AIP activation (AAC) control wells, respectively. NAC and AIP inactivation control (AIC) results were routinely comparable in all assays.

The second inhibition determination was on the basis of mean GFP fluorescence intensity (%I_MFI) as follows:

%I_MFI = 100 x [1 – (MFI_test – MFI_NAC)/(MFI_AAC – MFI_NAC)]

in which MFI_test, MFI_NAC and MFI_AAC represented the mean GFP fluorescence intensity of cells in wells containing test compound, the average MFI of cells in NAC wells and the average MFI of cells in AAC wells, respectively.

Compounds were considered active in the primary screening assay if they resulted in 50% or greater inhibition for both fluorescence parameters.

Activity Score Calculation. The activity scores of the active compounds were calculated as:

Activity Score = 100 x (% Inhibition - %I_GP_Min) / (%I_GP_Max - %I_GP_Min)

in which %I_GP_Min and %I_GP_Max were 50 and 100, respectively. The calculated number was rounded up to the next higher integer value to obtain the final reported score. An activity score of 0 was assigned to all other tested compounds.

Comments. There were a number of wells in which the number of bacteria detected during analysis was less than 25% of the average number detected over all wells of the plate. This suggested that test compounds in these wells may have been cytotoxic. The word "Cytotoxic" is entered in the column labeled PUBCHEM_ASSAYDATA_COMMENT to flag these compounds.

Center Summary of the Primary Screen

A flow cytometry based high throughput screening (HTS) campaign was undertaken to identify novel small molecule inhibitors of the quorum sensing pathway of *Staphylococcus aureus*. A set of 9,993 compounds, designated the 10K Set Type 1 (10KST1), and a separate set of 16,322 compounds, designated the 17K Set Type 1 (17KST1), was obtained from the Molecular Libraries Small Molecule Repository (MLSMR) maintained by Discovery Partners International in conjunction with the NIH Molecular Libraries Screening Center Network. There was an overlap of 2,595 compounds common to the two sets. An additional 367 compounds were cherry-picked from the remainder of the MLSMR compound collection on the basis of structural similarities with active compounds detected in the first two screening sets. The total number of unique compounds evaluated was 24,087. Hit selection criteria were satisfied by 64 compounds (0.3%).

Probe Optimization

Description of secondary screens used to optimize probe structure.

1. Dose Response Hit Confirmation (AID 700). The RNAIII promoter activation assay was used in a dose response format to further characterize the 64 hit compounds. For dose response curve application, test compound inhibition of AIP activity was calculated on the basis of mean GFP fluorescence intensity (%INHIB_MFI) as follows:

%INHIB_MFI = 100 x [1 – (MFI_test – MFI_NAC)/(MFI_AAC – MFI_NAC)]

in which MFI_test, MFI_NAC and MFI_AAC represented the mean GFP fluorescence intensity of cells in wells containing test compound, the average MFI of cells in NAC wells and the average MFI of cells in AAC wells, respectively.

Each compound was tested at 9 concentrations ranging from 3 nM to 20 µM. The concentration that inhibited activity by 50% (IC_{50}) was used as the basis of distinguishing active and inactive test compounds in the dose response assay. Test compounds with IC_{50} less than 10 µM were scored as active and all others as inactive. 19 compounds were confirmed as active by these criteria.

2. Primary screening of chemotype families in a structure activity relationship (SAR) series of compounds (AID 1014). Nine major chemotype families representing the most potent inhibitors were selected for follow up structure activity relationship (SAR) evaluation. A set of 240 compounds was selected by application of computational similarity search techniques to a subset of the ChemDiv collection of more than 1 million compounds. The compounds were purchased and screened in the RNAIII promoter activation assay in HTS format as described above. There were 32 compounds that satisfied hit selection criteria (13.3%).

3. Dose Response Confirmation of SAR Series Hits (AID 1015). The RNAIII promoter activation assay was used in a dose response format as described above to further characterize the 32 hit compounds. The 32 compounds represented 7 of the 9 tested chemotype families and all were confirmed as active. Nine of the active compounds were in Family 4, the chemotype family of the probe described in this report. The dose response profile of the probe **C094-0010** is illustrated in Fig. 1.

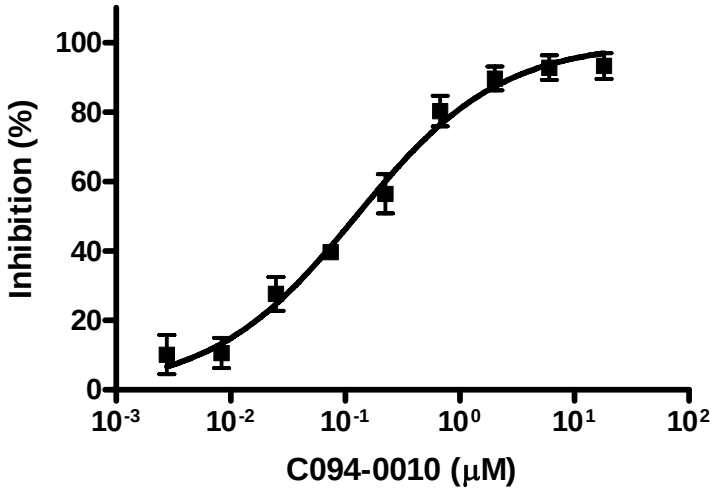


Fig. 1. Dose response profile of **C094-0010** in the RNAIII promoter activation assay. The assay was performed with *S. aureus* strain SA RN6390 (Agr1). IC_{50} = 0.125 μM in this experiment.

4. Viability Assay. An important characteristic of a quorum sensing pathway probe is that it not affect bacterial viability. *S. aureus* strain 1743 (Agr1) was therefore cultured for 3 hours in the presence and absence of **C094-0010**. There were 8.5 colony forming units (cfu)/ml in untreated cells, cells treated with **C094-0010**, as well as in cells treated with the related compound C094-0096 (SID 26746773). Thus, there were no detectable effects on the resulting viable colony counts. This was consistent with the lack of **C094-0010** effects on the numbers of bacteria detected in the flow cytometry based RNAIII promoter activation assay.

5. Virulence Factors Induced by AIP. Upregulation of RNAIII production leads *S. aureus* to switch to an invasive phenotype that secretes a variety of virulence factors such as α-hemolysin and lipase. α-hemolysin activity was measured in the supernatants of *S. aureus* MRSA 3757 (Agr1) and NM400 (Agr3) strains 4.5 hours after AIP treatment on the basis of rabbit erythrocyte lysis, as previously described (27,28) A hemolytic unit (HA_{50}) was defined as the amount of test solution able to liberate half of the total hemoglobin from the erythrocyte (28). In the presence of 12 μM **C094-0010**, a concentration that inhibited RNAIII

promoter activation in the MRSA 3757 Agr1 strain by 90% (data not shown), α-hemolysin activity was decreased by less than 20% (Table 1). By contrast, **C094-0010** promoted a more than 50% decrease in α-hemolysin production by the NM400 Agr3 strain (Table 1).

Table 1. Inhibition of AIP-induced α-hemolysin production by **C094-0010**.

	MRSA 3757 (Agr1)		NM400 (Agr3)	
Treatment	HA_{50}	Inhibition (%)	HA_{50}	Inhibition (%)
None	2.0		0.03	
AIP	48.6		1.90	
AIP + C094-0010	39.4	19.7	0.83	57.2
AIP + C094-0096	41.7	14.8	0.88	54.6

Lipase (glycerol ester hydrolase) production was evaluated by monitoring the lipolytic kinetics of an overnight culture supernatant on tributyrin emulsion at wavelength 450nm as previously described (29). AIP-induced lipase activity was substantially reduced by **C094-0010** in the NM400 Agr3 strain (Fig. 2, right) but was unaffected in the MRSA 3757 Agr1 strain (Fig. 2, left). Thus, **C094-0010** selectively inhibited lipase production and promoted ~2.5-fold greater inhibition of α-hemolysin production in the Agr3 as compared to the Agr1 strain of *S. aureus*. A similar pattern of inhibition was produced by the related compound C094-0096 (Table 1, Fig. 2).

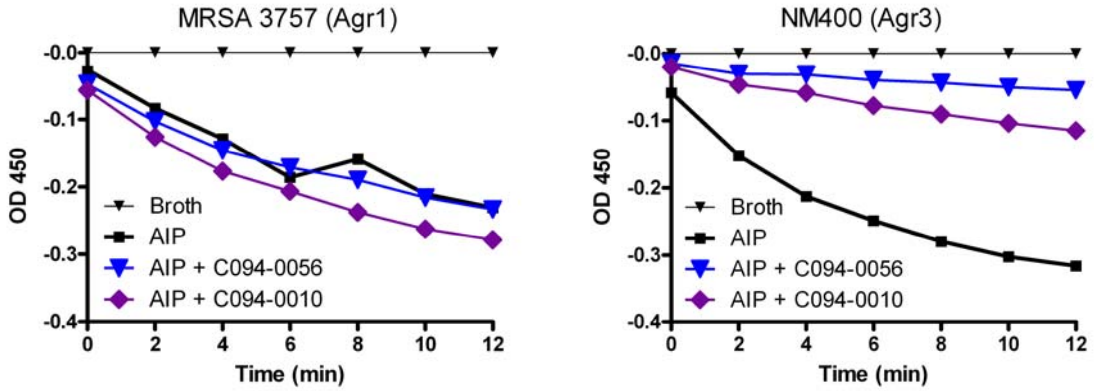


Fig. 2. Inhibition of AIP-induced lipase production by **C094-0010**.

6. FITC-AIP Binding Assay. To determine effects of **C094-0010** on AIP binding to its AgrC surface receptor, a fluorescein-conjugate of AIP (FITC-AIP) was prepared. *S. aureus* cultures were incubated with 1 μM FITC-AIP for 1 h at 37ºC in the presence and absence of test compounds. AIP-FITC binding was only reduced ~26% by **C094-0010** in the NM300 Agr1 strain of *S. aureus* (Table 2). The related compound C094-0096 inhibited only ~20%. By contrast, 6 μM benzbromarone resulted in ~70% inhibition of FITC-AIP binding. All compounds were tested at concentrations that consistently reduced RNAIII promoter activation by 90% or more (data not shown). Thus, the minimal effects of **C094-0010** and C094-0096 on FITC-AIP binding suggest their RNAIII promoter inhibitory effects to be mediated in a signaling pathway downstream of AIP binding. The Agr3 strain specific effects on virulence factor production were also consistent with this notion (Table 1, Fig. 2). Benzbromarone has been separately reported by our screening center as an inhibitor of AIP binding.

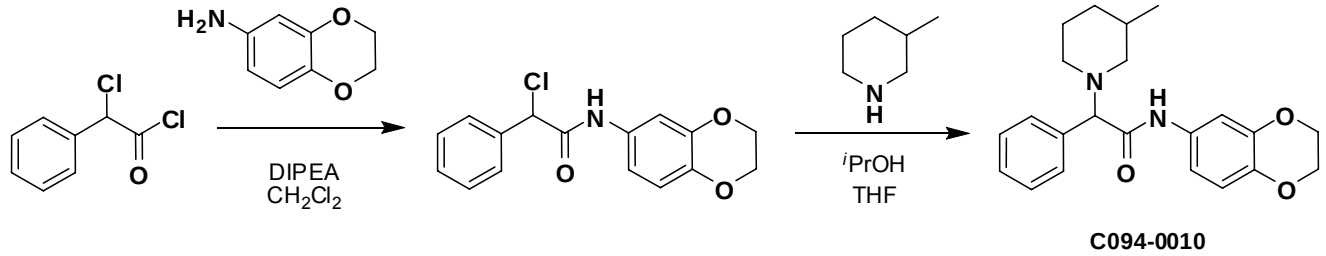
Table 2. Effect of probes on binding of FITC-AIP in NM300 (Agr1) *S aureus*.

Treatment	Mean Channel Fluorescence Intensity	Inhibition (%)
Broth	4.7	
FITC-AIP	77.9	
FITC-AIP + Benzbromarone	27.6	68.7
FITC-AIP + C094-0010	59.0	25.8
FITC-AIP + C094-0096	63.5	19.7

<u>Center comments on chemistry strategy leading to probe identification</u>:

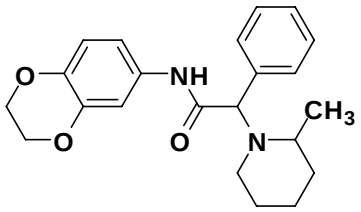
Synthetic Pathway for C094-0010

The probe was synthesized in two steps from the α-chloro-benzeneacetyl chloride starting material. The acetyl chloride was first coupled with 1,4-Benzodioxan-6-amine followed by the alpha-halo substitution of the intermediate with 3-methyl-piperidine to afford C094-0010.



Note: **C094-0010** is an equimolar mixture of two diastereomers, each racemic.

Characterization Data for C094-0010



Formula: $C_{22}H_{26}N_2O_3$
FW: 366.45

C094-0010: ^{1}H NMR (Fig. 3, 400 MHz, DMSO-d6) δ 0.74 (d, 6 H), 0.81-0.91 (m, 3 H), 1.36-1.83 (m, 12 H), 1.93-2.13 (m, 2 H), 2.40-2.59 (m, 15 H), 2.77-2.92 (m, 2 H), 3.25-3.43 (m, 25 H), 3.83-3.97 (m, 1 H), 4.11-4.23 (m, 7 H), 6.66-6.78 (m, 2 H), 6.99 (dt, *J* = 8.89, 1.12 Hz, 2 H), 7.22-7.37 (m, 7H), 7.39-7.49 (m, 4 H), 9.75-9.89 (m, 2 H); ^{13}C NMR (Fig. 3, 100 MHz, $CDCl_3$) δ 143.5, 131.7, 129.2,

128.4, 128.1, 117.2, 112.9, 109.0, 64.5, 64.3, 58.1, 53.7, 31.5, 19.4; MS (M+H) 367; purity was assessed at 92.5% by analytical HPLC (Fig. 4).

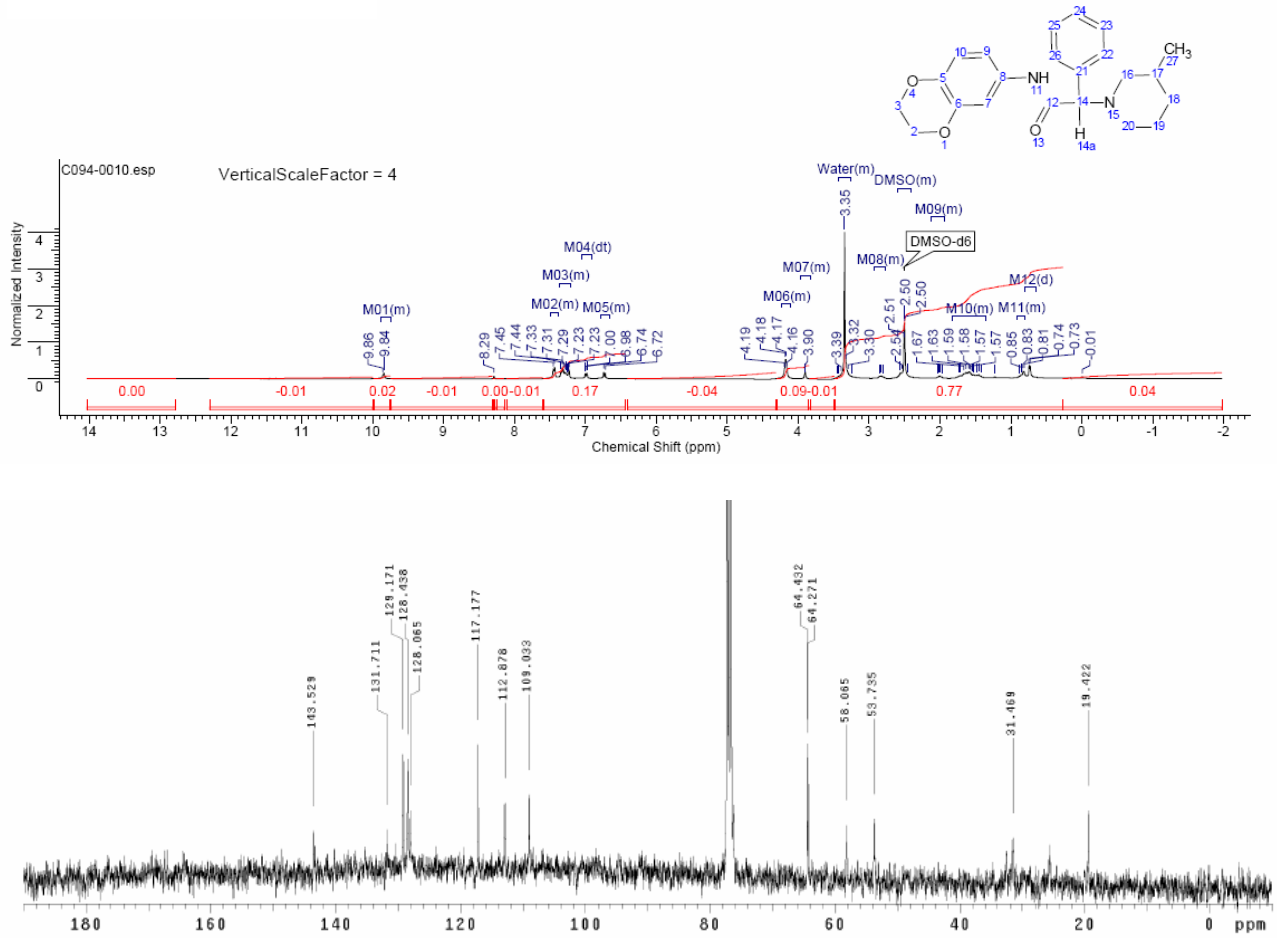
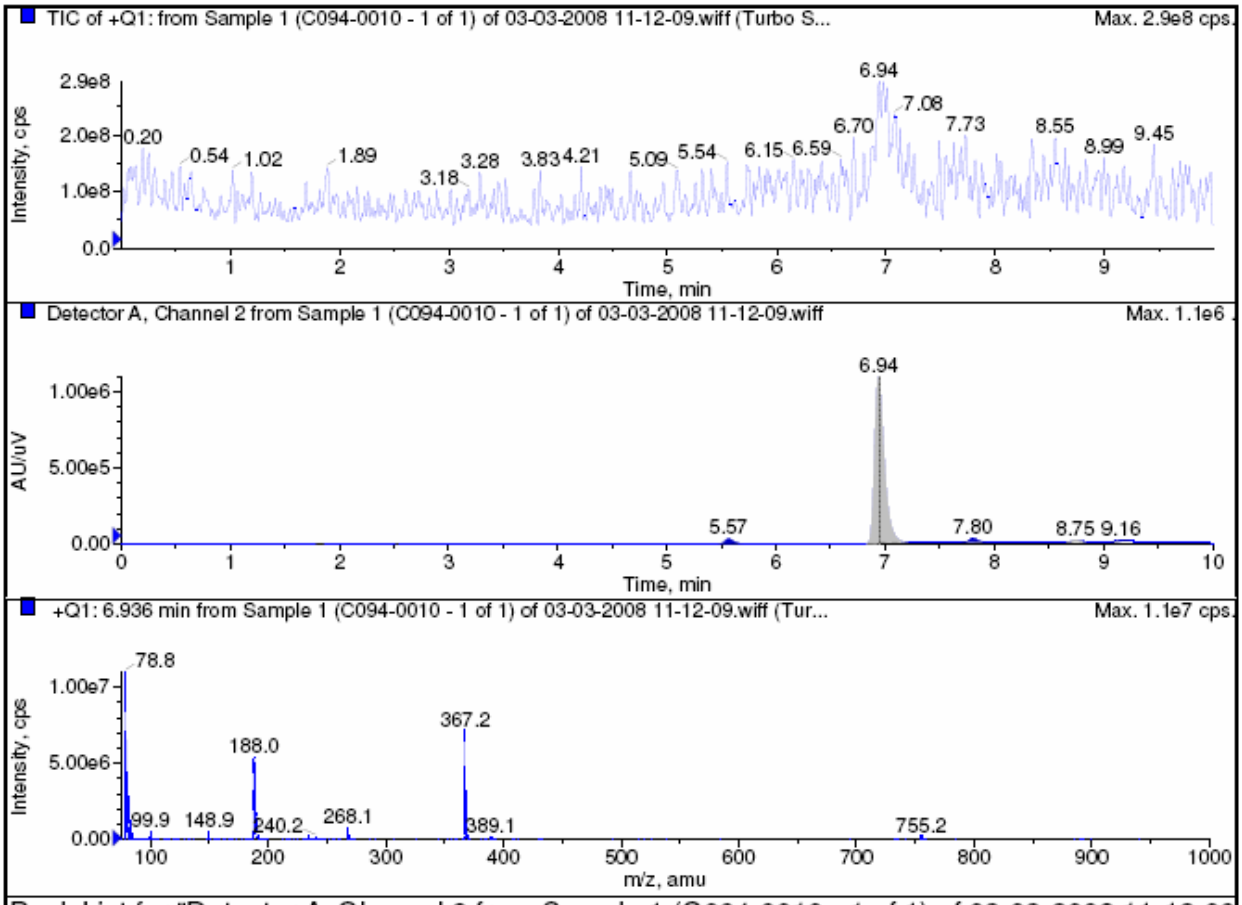


Fig. 3. ^{1}H NMR (top) and ^{13}C NMR (bottom) of **C094-0010**.



Peak List for "Detector A, Channel 2 from Sample 1 (C094-0010 - 1 of 1) of 03-03-2008 11-12-09.wiff

	Time (min)	Area (counts)	% Area	Height	% Height	Width (min)
1	5.5656	1.8084e5	2.4908	3.3873e4	2.8454	0.2167
2	6.9388	6.7892e6	93.5101	1.1012e6	92.5013	0.3750
3	7.8037	1.6720e5	2.3029	3.0438e4	2.5569	0.2250
4	8.7454	6.6470e4	0.9155	1.3480e4	1.1323	0.1750
5	9.1601	5.6681e4	0.7807	1.1477e4	0.9641	0.1750

Analyst Version: 1.4.2 Sample Comment: Collected by: I-XP-NEWLCMS\lcms

Figure 4. LC-MS data for C094-0010.

Center summary of probe properties and recommendations for the scientific use of probe as research tool.

C094-0010 is a novel inhibitor of the RNAIII promoter activation response induced in *S. aureus* by AIP. The IC_{50} for inhibition of RNAIII promoter activation is in the range of 100 – 200 nM and maximal inhibition (90% or more) can be achieved at 12 μM concentrations in *in vitro* experimental systems. Under such conditions it inhibits AIP-induced production of virulence factors α-hemolysin and lipase in Agr3 but not Agr1 strains of *S. aureus*. Receptor binding studies with FITC-AIP indicate that it does not significantly inhibit the binding of AIP to the AgrC cellular receptor, the initial step in the bacterial quorum sensing pathway. Cell viability is unaffected under these conditions for up to 3 hours at 37ºC. **C094-0010** is recommended as a probe of AIP-induced signaling pathways in strains of *S. aureus* expressing the Agr3 variant of the agr locus. The specific pathway element(s) targeted by probe **C094-0010** remain to be determined.

Appendices

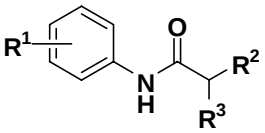
<u>Comparative data on probe, similar compound structures and prior probes.</u>

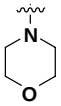
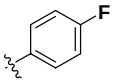
Structure Activity Relationship (SAR) Evaluation

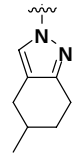
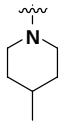
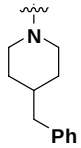
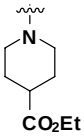
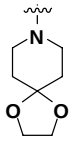
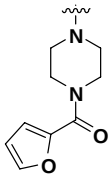
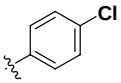
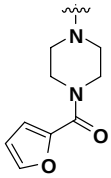
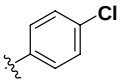
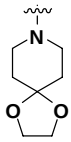
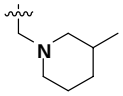
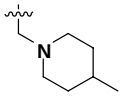
This family was based on a series of aryl carboxamides containing either 1,3-benzodioxole or benzo-1,4-dioxane substituents. The first set of analogs maintained these moieties at the N-substitution of the amide. The best inhibitions were seen in this sub-group. With the exception of Entry 1, the 1,3-benzodioxoles were generally more active, particularly when R^2 was a simple phenyl and R^3 was a piperidine (Table 1). For the structural outlier, Entry 1, even small modifications yielded completely inactive compounds (Entries 2-4). In comparison to the active 1,3-benzodioxole series (Entries 5-8), N-heterocyclic R^2 substituents containing ethers (Entry 9), esters (Entry 10), ketal protections (Entries 11 and 14), or ketones (Entries 12 and 13) lead to loss of inhibition. Generally, having anything at R^2 other than a simply phenyl seems to be problematic. The contradictory examples to this include Entry 24 which is unsubstituted at R^2 and Entries 28, 29, and 40 in which the 1,3-benzodioxole is C-substituted on the amide at R^2.

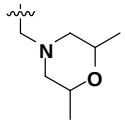
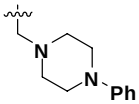
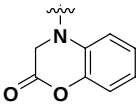
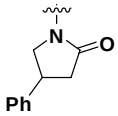
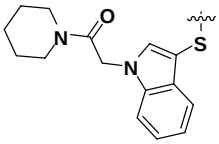
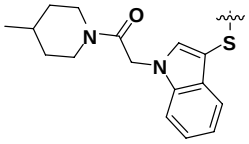
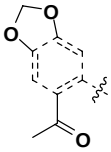
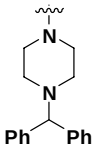
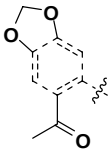
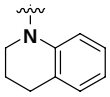
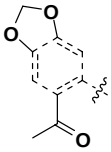
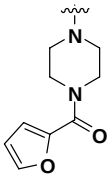
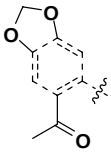
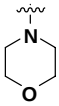
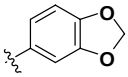
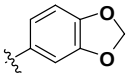
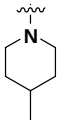
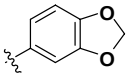
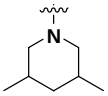
In this subgrouping, R^1 was a simply substituted aryl and R^3 was consistently a piperidine. The spatial morphology of the two connectivity-defined subgroups are still very similar to one another. Entry 45 shows a third case in which the 1,3-benzodioxole is attached through R^3, but the connectivity results in a different morphology and consequently inactivity. Extra aryl substituents on the 1,3-benzodioxoles (Entries 25-27, 35-39, and 41-43) seem to lead to inactivity as well, with the exception being Entries 24 and 40. Increased complexity in the substituents (i.e. Entries 12, 13, and 19-23) also appeared detrimental to inhibition.

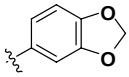
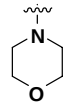
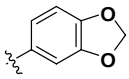
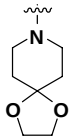
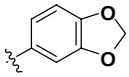
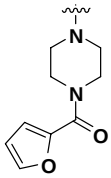
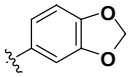
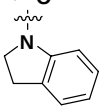
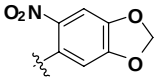
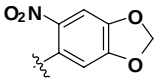
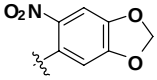
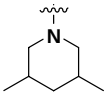
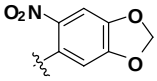
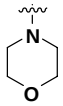
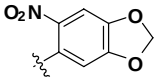
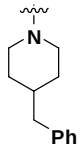
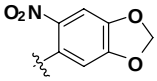
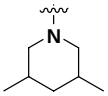
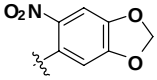
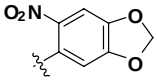
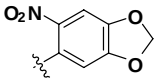
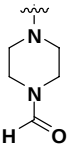
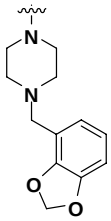
Table 1.



Entry	ID (SID)	R^1	R^2	R^3	Activity (μM)
1	C094-0010 (26746770)	3,4-fused	Ph		0.13
2	C094-0001 (26746768)	3,4-fused	Ph		-
3	C094-0004 (26746769)	3,4-fused			-

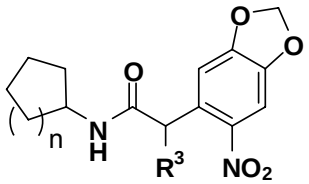
4	C881-0742 (26746847)	3,4-fused	H		-
5	C094-0056 (26746773)	3,4-fused	Ph		0.16
6	C094-0062 (26746774)	3,4-fused	Ph		0.98
7	C094-0049 (26746771)	3,4-fused	Ph		1.26
8	C094-0066 (26746776)	3,4-fused	Ph		0.80
9					
10	C094-0055 (26746772)	3,4-fused	Ph		-
11	C094-0064 (26746775)	3,4-fused	Ph		-
12	C094-0068 (26746777)	3,4-fused	Ph		-
13	C094-0079 (26746778)	3,4-fused			-
14	C094-0082 (26746779)	3,4-fused			-
15	4239-0120 (26746713)	3,4-fused	H		-
16	4239-0104 (26746712)	3,4-fused	H		-
17					

18	4239-0123 (26746678)	3,4-fused	H		-
19	4239-0130 (26746689)	3,4-fused	H		-
20	7706-0501 (26746748)	3,4-fused	H		-
21	C301-1977 (26746811)	3,4-fused	H		-
22	E587-0232 (26746892)	3,4-fused	H		-
23	E587-0523 (26746893)	3,4-fused	H		-
24	6208-0484 (26746734)		H		1.51
25	6208-0479 (26746733)		H		-
26	6208-0487 (26746671)		H		-
27	8014-2372 (26746685)		H		-
28	C094-0108 (26746780)	4-^{i}Pr			0.25
29	C094-0121 (26746783)	4-^{i}Pr			0.72
30	C094-0126 (26746785)	4-^{i}Pr			-

31	C094-0109 (26746781)	4-^{i}Pr			-
32	C094-0123 (26746784)	4-^{i}Pr			-
33	C094-0127 (26746786)	4-^{i}Pr			-
34	C094-0128 (26746787)	4-^{i}Pr			-
35	C094-0170 (26746665)	4-^{i}Pr			-
36	C094-0157 (26746664)	4-^{i}Pr			-
37	C094-0174 (26746667)	4-^{i}Pr			-
38	C094-0156 (26746789)	4-^{i}Pr			-
39	C094-0173 (26746666)	4-^{i}Pr			-
40	C094-0151 (26746663)	4-MeO			1.31
41	C094-0133 (26746661)	4-MeO			-
42	C094-0147 (26746662)	4-MeO			-
43	C094-0148 (26746788)	4-MeO			-
44	4038-0190 (26746709)	4-Me	H		-

Several similarity search analogs were also identified, however none of them showed any inhibition. Over a dozen analogs with modifications to the region expressing R^1 that did not contain an aryl moiety were also shown to be inactive. Figure 1 illustrates the scaffold with 5 and 6 membered alkyl rings in place of that aryl group. The R^3 group was varied similarly as shown in Table 1 with a mixture of piperidine and piperizine substituents while R^2 was held constant as the 5-nitro-1,3-benzodioxole. However, the fact that only one of the nitro-benzodioxole analogs showed activity (as apposed to 8 non-nitro) dictates that not necessarily all alkyl carboxamide derivatives are inactive, it only means that this particular analog series is inactive.

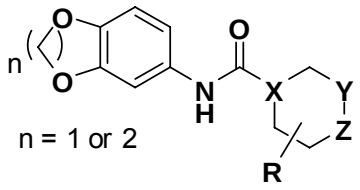
Figure 1.



n = 1 or 2

Another dozen compounds with ring-fused R^2/R^3 regions were also observed to be inactive. Figure 2 shows the variety of configurations represented in these analogs. Both the 1,3-benzodioxole and the benzo-1,4-dioxane aryl groups were represented in R^1. More directly analogous structures were represented where X = CH, and Z and Y = CH or N (Z and Y were not N simultaneously). The closely related urea analogs were represented when X = N. Despite the variety of functional groups, connectivity, and substitution no activity range can be reported for this set of analogs either.

Figure 2.



n = 1 or 2

Comparative data showing probe specificity for target

C094-0010 has been analyzed in 202 assays published on the PubChem website and confirmed to be active in only 1 (Table 2), the bacterial quorum sensing SAR dose response assay described in this report.

Table 2. **C094-0010** activity reported in confirmation assays

AID	Active	Discrepant	Outcome Method	Name
1015	1		Confirmatory	Inhibitors of Bacterial Quorum Sensing Structure Activity Relationship (SAR) Analysis: Dose Response Assay

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