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(54) **Title**: COMPOSITION AND METHOD FOR PREVENTION, MITIGATION OR TREATMENT OF AN ENTEROPATHOGENIC BACTERIAL INFECTION

(57) **Abstract**: Provided herein are bicyclic compounds for decreasing the expression of bacterial virulence factors thereby preventing, mitigating, or treating bacterial infection,

COMPOSITION AND METHOD FOR PREVENTION, MITIGATION OR TREATMENT OF AN ENTEROPATHOGENIC BACTERIAL INFECTION

RELATED APPLICATIONS

[0001] This application is a continuation-in-part application of U.S. Patent Application Serial No. 14/837,139 filed August 27, 2015, which is a continuation-in-part of U.S. Patent Application Serial No. 13/384,860 filed February 23, 2012, which claims benefit of priority to PCT/US2010/042704 filed July 21, 2010 and U.S. Provisional Application Serial Nos. 61/301,264 filed February 4, 2010 and 61/227,190 filed July 21, 2009, the contents of which are incorporated herein by reference in their entireties.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under contract numbers AI072661 and AI039654 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] The increasing resistance of bacterial pathogens to antibiotics, combined with fundamental advances in understanding the mechanisms and regulation of bacterial virulence, has prompted the identification of pathogen anti-virulence drugs that antagonize the activity of virulence factors. Cholera is an acute intestinal infection caused by the bacterium *Vibrio cholerae,* a gram-negative flagellated bacillus. In addition to being a class B bioterrorism threat, cholera is more widespread today than it was in the previous century. The expression of *V. cholerae's* primary virulence factors, the toxin-coregulated pilus (TCP) and cholera toxin (CT), occurs via a transcriptional cascade involving several activator proteins, and serves as a paradigm for the regulation of bacterial virulence. Strains of *V. cholerae* capable of causing the significant epidemics and pandemics of cholera that have occurred throughout history possess two genetic elements, the Vibrio pathogenicity island (VPI) and the lysogenic CTX phage. Both of these elements have inserted into the circular chromosome I and are present in the

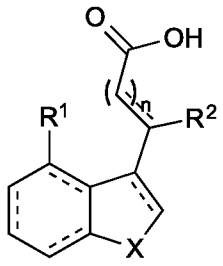
pathogenic forms of the organism. The VPI contains the genes responsible for the synthesis and assembly of the essential colonization factor TCP, and the CTX phage encodes the CT genes. Expression of the TCP and CT genes is coordinately regulated at the transcriptional level by a virulence cascade involving activator proteins encoded both within the VPI and the ancestral genome. AphA and AphB initiate the expression of the cascade by a novel interaction at the *tcpPH* promoter. AphA is a member of a new regulator family and AphB is a LysR-type activator, one of the largest transcriptional regulatory families. Once expressed, cooperation between TcpP/TcpH and the homologous transmembrane activators ToxR/ToxS activates the *toxT* promoter. ToxT, an AraC/XylS (A/X) type regulator, then directly activates the promoters of the primary virulence factors. Thus, ToxT is the paramount regulator of virulence gene expression.

[0004] ToxT inhibitors have been identified and shown to provide protection against intestinal colonization by *V. cholerae*. For example, bile (Schuhmacher, et al. (1999) *J. Bacteriol.* 181:1508-14) and several of its unsaturated fatty acid constituents, *i.e.,* oleic acid, linoleic acid, and arachidonic acid (Chatterjee, et al. (2007) *Infect. Immun.* 75:1946-53) have been shown to inhibit virulence factor gene expression. Similarly, virstatin, a small molecule 4-[N-(1,8-naphthalimide)]-n-butyric acid, has been shown to inhibit virulence regulation in *V. cholerae* (Hung, et al. (2005) *Science* 310(5748):670-4). Further, US 5,866,150 teaches compounds having the structure: $CH_3(CH_2)_n$-CH=CH-CH_2CH=CH-$(CH_2)_n$-R-COOR' for use in treating bacterial infections including, *e.g., S. aureus, V. cholera, S. dysenteria, B. substilis,* and *S. typhemurium.*

[0005] High resolution structure of ToxT has shown that ToxT contains an almost completely buried and solvent inaccessible sixteen carbon fatty acid bound to a pocket in the N-terminal domain, which can influence its DNA binding activity. In particular, virulence gene expression can be reduced between 6-8 fold with *cis*-palmitoleic acid and 10-15 fold with oleic acid (Lowden, et al. (2010) *Proc. Natl. Acad. Sci. USA* 107:2860-5).

SUMMARY

[0006] This disclosure provides a compound having the structure of Formula I, or a hydrate, isomer, prodrug or pharmaceutically acceptable salt of Formula I:



I

wherein R^1 is C_1-C_6 alkyl, C_1-C_6 alkenyl, C_4-C_6 aryl, halo, -CF_3, -OCH_3, -NO_2, -CN, -OH, -NMe_2, -COOH, or -$COOCH_3$; R^2 is hydrogen, alkylidenyl, or oxo; X is –CH_2-CH_2-, –CH=CH-, -NH-, -CH=N-, -N=CH-, -CH_2-NH- or -NH-CH_2-; n is 0-6; and dashed lines represent bonds that are independently present or absent. A pharmaceutical, nutraceutical, nutritional, medical nutrition food or functional food composition containing a compound of Formula I is also provided.

[0007] In certain aspects, provided herein are methods for decreasing expression of a bacterial virulence factor and preventing, mitigating, or treating an infection by a bacterium that expresses an A/X regulatory protein, comprising contacting the bacterium that expresses an A/X regulatory protein with a disclosed compound, such that the expression of a virulence factor by said bacterium is decreased. In some embodiments the bacterium is *Vibrio cholerae, Escherichia coli*, *Shigella flexneri*, *Yersinia enterocolitica*, *Yersinia pestis, Brucella abortus Salmonella typhi*, *Bacillus anthracis, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus* or *Salmonella typhimurium.*

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figure 1 shows a sequence alignment of A/X family members from *V. cholerae* (Vc ToxT; SEQ ID NO:1), an *E. coli* ETEC strain (Ec FapR; SEQ ID NO:2), an *E. coli* EPEC strain (Ec PerA; SEQ ID NO:3), *S. typhi* (St SirC; SEQ ID NO:4), *S. typhimurium* (St HilD; SEQ ID NO:5), *S. flexneri* (Sf VirF; SEQ ID NO:6), *Y. enterocolitica* (Ye VirF; SEQ ID NO:7), an *E. coli* ETEC strain (Ec Rns; SEQ ID NO:8), *Y. pestis* (Yp AraC; SEQ ID NO:9), *B. anthracis* (Ban AraC; SEQ ID NO:10), *B. abortus* (Bab AraC; SEQ ID NO:11) and *C. botulinum* (Cb AraC; SEQ ID NO:12). Secondary

structure predictions and homology modeling indicate common helix (H), loop/turn (L) and lysines or other positive amino acids (underlined residues) at positions homologous to those identified in ToxT. The entire sequence lengths were compared, but only partial sequences are shown here.

[0009] Figure 2 shows the effects of compound **1** on *tcp* expression. *V. cholerae* cells were grown in LB pH 6.5 at 30°C for 18 hours ± the indicated inhibitor in methanol. β-galactosidase activity of a tcp-lacZ reporter construct was determined. β-galactosidase units are shown for a wild-type strain treated with methanol (MeOH), 0.01% oleic acid (0.01% OA), 0.02% oleic acid (0.02% OA), or 0.02% compound **1** (Comp**1**) as compared to an untreated control (ToxT) and an untreated ToxT deletion strain (ΔToxT).

[0010] Figure 3 shows the fatty acid binding region of ToxT. The carboxylate head of *cis*-palmitoleate (green) interacts with Lys31 of the N-terminal domain (blue) and Lys230 of the C-terminal domain (grey). Full structure (inset) obtained from PDB 3GBG.

[0011] Figures 4A-B shows that small molecule inhibitors maintain a fatty acid-like structure. General structure of a compound disclosed herein (Figure 4A) compared to the bound conformation of cis-palmitoleate (Figure 4B).

[0012] Figures 5A-C show the inhibition of *tcpA* expression and autoagglutination activities by compounds disclosed herein. Figure 5A: β-galactosidase activity of tcpA-lacZ fusion construct in the presence of virstatin (vir.), ten synthesized compounds, oleic acid (OA) and palmitoleic acid (POA) at concentrations of 5 μM (blue) and 0.5 μM (grey), graphed as percent change. ΔToxT is calculated based on 100% WT. The rest are calculated based on 100% WT+DMSO. Figure 5B: Western blot showing TcpA expression in the corresponding lanes. Compounds were added to a final concentration of 5μM. Fatty acids were not tested. Top non-specific band is a loading control. Figure 5C: Autoagglutination of O395 cultures grown in the presence of 0.5 μM (top) and 0.05 μM (bottom) compounds 4a, 5a, 3b, and 4b.

[0013] Figures 6A-C show inhibition of ToxT-DNA binding interactions by compounds disclosed herein. Figure 6A: ToxT EMSA in the presence of virstatin and our compounds. All lanes contain a DIG labeled, 84-bp segment of the *tcpA* promoter. All lanes except lane 1 contain 0.78 μM ToxT. The solvent (DMSO) does not inhibit DNA

binding (lane 3). An excess of the unlabeled DNA segment competes for binding (lane 4). The presence of 100 μM virstatin (vir) or 100 μM synthesized compounds inhibits DNA binding. Figure 6B: ToxT does not shift the negative control DIG CJ2.6 DNA, a mutated segment of the tcpA promoter which ToxT cannot bind. Figure 6B: ToxT EMSA in the presence of virstatin and four lead compounds. All lanes contain labeled DNA. Virstatin is tested at 100, 10, 1, and 0.1 μM concentrations. Compounds 4a, 5a, 3b and 4b are each tested 10, 1, and 0.1 μM.

[0014] Figures 7A-C show X-ray crystal structures of ToxT-inhibitor complexes reveal compounds displace the fatty acid and bind in the regulatory pocket. Figure 7A: Simulated annealing $F_o - F_c$ omit maps of ToxT bound to compounds 3b (left) and 5a (right) contoured at 2.5 σ. Figure 7B: Overlay of crystal structures of ToxT bound to fatty acid (PDB: 3GBG) with ToxT bound to 3b (left) and ToxT bound to 5a (right). Figure 7C: Overlay of crystal structures of ToxT bound to 3b (left) and ToxT bound to 5a (right) with the conformations predicted by Autodock. Autodock predictions are purple and orange, respectively.

[0015] Figures 8A-C show competition STD NMR of compounds 5a and 3b in the presence of virstatin. Figure 8A: 1H-NMR spectrum of virstatin alone (top) and in the presence of 20 μM ToxT (middle). The corresponding STD-difference spectrum resulting from on-resonance saturation (irradiation of ToxT) and the transfer of resonance to virstatin (bottom). Figure 8B, Figure 8C: 1H-NMR spectrum of 100 μM virstatin in the presence of 20 μM ToxT (Figures 8B and 8C top) and with the addition of compound 3b (Figure 8B middle) or compound 5a (Figure 8C middle). Competition STD-difference spectrum showing the reduction in STD signal intensities of virstatin due to competitors 3b (Figure 8B bottom) and 5a (Figure 8C bottom). Structures of virstatin, 3b, and 5a with aromatic protons labeled shown to the right of each corresponding spectrum.

[0016] Figures 9A-B show that unsaturated fatty acids do not inhibit autoagglutination. Figure 9A: Autoagglutination of O395 cultures grown in the presence of 0.5 μM, 0.05 μM, and 5 nM virstatin and compounds 4a, 5a, 3b, and 4b. Figure 9B: Cultures containing 0.5 μM and 0.05 μM oleic and palmitoleic acids were grown similarly; the unsaturated fatty acids were not soluble at 5 nM.

[0017]

[0018] Figure 10 shows the fatty acid binding region of ToxT comparing *cis*-palmitoleate (green, from crystal structure 3GBG) and models of compounds 3a, 3b, 3c, 3d, 4a, 4b, 4c, 5a, 5b, 5c and virstatin. The naphthalenes with double bonds in the carboxylate chain are dark blue, the naphthalenes with single bonds are medium blue, tetralins are orange, and virstatin is magenta. Bound conformations were predicted using Autodock.

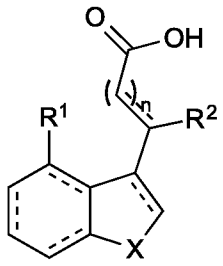
[0019] Figure 11 shows an electron density map of the natural fatty acid ligand. Simulated annealing Fo – Fc omit map of ToxT bound to cis-palmitoleate contoured at 2.5 σ (PDB: 3GBG).

[0020] Figure 12 shows the STD-AF_0 curve of virstatin as a function of ligand concentration. Data were fit to equation 3. The isotherm was constructed using the STD data from protons $H_{e,f}$ of virstatin. The same was done for protons $H_{a,b}$ (data not shown).

DETAILED DESCRIPTION

[0021] A class of bicyclic compounds has now been identified, which exhibits anti-virulence activity against *V. cholerae*. The compounds of this invention were designed to bind to the pocket located in the N-terminus of ToxT (Lowden, et al. (2010) *Proc. Natl. Acad. Sci. USA* 107:2860-5), thereby disrupting DNA binding activity and virulence gene expression. Given that ToxT homologues are found in a wide variety bacterial pathogens, the compounds of this invention find use as broad spectrum anti-virulence agents in the treatment of antibiotic-resistant bacterial infections as well as in prophylactic treatment of infections, *e.g.*, for travelers or military personnel in areas with suboptimal water and/or food quality. Moreover, because the compounds of this invention are highly specific for pathogens, the normal bacterial flora of the gut is not affected.

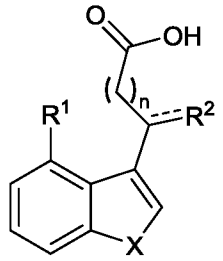
[0022] Compounds provided herein may have the structure of Formula I, which includes hydrates, isomers, prodrugs or pharmaceutically acceptable salts of Formula I:



Formula I

wherein R^1 is hydrogen (H) or C_1-C_6 alkyl, C_1-C_6 cycloalkyl, alkyl C_1-C_6 alkenyl, C_4-C_6 aryl, halo (*e.g.*, F, Cl, Br or I), $-CF_3$, $-OCH_3$, $-NO_2$, -CN, -OH, $-NMe_2$, -COOH, or $-COOCH_3$; R^2 is hydrogen (H), alkylidenyl ($=CH_2$), or oxo (=O); X is $–CH_2CH_2-$, –CH=CH-, -NH-, -C=NH-, -N=C-, $-CH_2$-NH- or -NH-CH_2-; n is 0-6; and dashed lines represent bonds that are independently present or absent. In certain embodiments, n is 3, 4, or 5.

[0023] In one embodiment, the compound of Formula I has the structure of Formula Ia:



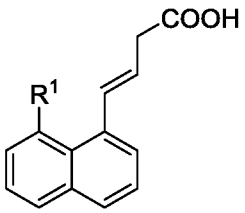
Ia

or a pharmaceutically acceptable salt thereof, wherein R^1 is selected from the group consisting of hydrogen, -OH, and C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group; R^2 is hydrogen, attached by a single bond, or R^2 is $=CH_2$ or =O, attached by a double bond; X is selected from the group consisting of –CH=CH-, -NH-, -CH=N-, and -N=CH-; n is selected from 1, 2, 3 and 4; and a dashed line represents a bond that is either present or absent.

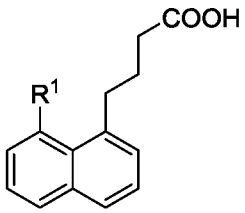
[0024] In a particular embodiment of Formula Ia, R^1 is C_1-C_6 alkyl. In another particular embodiment, R^1 is methyl. In another particular embodiment, R^1 is ethyl. In another particular embodiment, R^1 is propyl. In another particular embodiment, R^1 is n-butyl. In another particular embodiment, R^1 is t-butyl. In another particular

embodiment, R^1 is pentyl. In another particular embodiment, R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is substituted with an –OH group. In another particular embodiment, R^1 is $–CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2$ CH_2CH_2OH. In another particular embodiment, R^1 is hydroxyl. In another particular embodiment, X is –CH=CH-. In another particular embodiment, X is -NH-. In another particular embodiment, X is -CH=N-. In another particular embodiment, X is -N=CH-. In another particular embodiment, R^2 is $=CH_2$. In another particular embodiment, R^2 is =O. In another particular embodiment, n is 1. In another particular embodiment, n is 2. In another particular embodiment, n is 3. In another particular embodiment, n is 4.

[0025] In an embodiment, the compound of Formula Ia is selected from the following:



and



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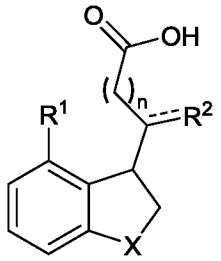
Ia01 Ia02

wherein R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group. In a particular embodiment, R^1 is selected from the group consisting of methyl, ethyl, propyl, butyl, pentyl, hexyl, and isomers thereof. In another particular embodiment, R^1 is selected from the group consisting of methyl, ethyl, propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, and neopentyl In another particular embodiment, R^1 is $–CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2$ CH_2CH_2OH.

[0026] In another embodiment, the compound of Formula I has the structure of Formula Ib:

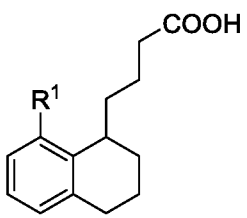


Ib

or a pharmaceutically acceptable salt thereof, wherein R^1 is selected from the group consisting of hydrogen, hydroxyl and C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group; R^2 is hydrogen, attached by a single bond, or R^2 is $=CH_2$ or =O, attached by a double bond; X is selected from the group consisting of -NH-, -CH_2-CH_2-, -CH_2-NH- and -NH-CH_2-; n is selected from 1, 2, 3 and 4; and a dashed line represents a bond that is either present or absent.

[0027] In a particular embodiment of Formula Ib, R^1 is C_1-C_6 alkyl. In another particular embodiment, R^1 is methyl. In another particular embodiment, R^1 is ethyl. In another particular embodiment, R^1 is propyl. In another particular embodiment, R^1 is n-butyl. In another particular embodiment, R^1 is t-butyl. In another particular embodiment, R^1 is pentyl. In another particular embodiment, R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is substituted with an –OH group. In another particular embodiment, R^1 is $–CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2CH_2CH_2OH$. In another particular embodiment, R^1 is hydroxyl. In another particular embodiment, X is -CH_2-CH_2-. In another particular embodiment, R^2 is hydrogen. In another particular embodiment, R^2 is $=CH_2$. In another particular embodiment, R^2 is =O. In another particular embodiment, n is 1. In another particular embodiment, n is 2.

[0028] In an embodiment, the compound of Formula Ib has the structure of Formula Ib01:



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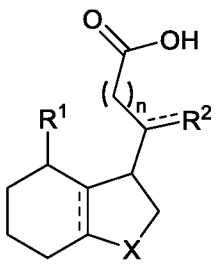
Ib01

wherein R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group. In a particular embodiment, R^1 is selected from the group consisting of methyl, ethyl, propyl, butyl, pentyl, hexyl, and isomers thereof. In another particular embodiment, R^1 is selected from the group consisting of methyl, ethyl, propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, and neopentyl. In another particular embodiment, R^1 is $–CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2$ CH_2CH_2OH.

[0029] In another embodiment, the compound of Formula I has the structure of Formula Ic:



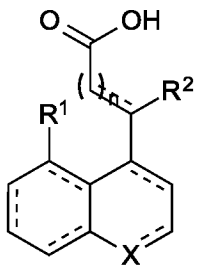
Ic

or a pharmaceutically acceptable salt thereof, wherein R^1 is selected from the group consisting of hydrogen, hydroxyl and C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group; R^2 is hydrogen, attached by a single bond, or R^2 is $=CH_2$ or =O, attached by a double bond; X is $-CH_2-CH_2-$; n is selected from 1, 2, 3 and 4; and each dashed line independently represents a bond that is either present or absent.

[0030] In a particular embodiment of Formula Ic, R^1 is C_1-C_6 alkyl. In another particular embodiment, R^1 is methyl. In another particular embodiment, R^1 is ethyl. In another particular embodiment, R^1 is propyl. In another particular embodiment, R^1 is n-butyl. In another particular embodiment, R^1 is t-butyl. In another particular embodiment, R^1 is pentyl. In another particular embodiment, R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is substituted with an –OH group. In another particular embodiment, R^1 is $–CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2CH_2OH$. In another particular embodiment, R^1 is $–CH_2CH_2$ CH_2CH_2OH. In another particular embodiment, R^1 is hydroxyl. In another particular

embodiment, R^2 is hydrogen. In another particular embodiment, R^2 is $=CH_2$. In another particular embodiment, R^2 is =O. In another particular embodiment, n is 1. In another particular embodiment, n is 2. In another particular embodiment, n is 3. In another particular embodiment, the dashed line indicates a bond that is present (i.e., indicating a double bond). In another particular embodiment, the dashed line indicates a bond that is absent (i.e., indicating a single bond).

[0031] In some embodiments, the invention is a compound having the structure of Formula II, which includes hydrates, isomers, prodrugs or pharmaceutically acceptable salts of Formula II:

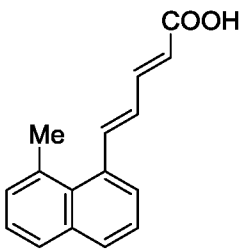


Formula II

wherein R^1 is selected from the group consisting of C_1-C_6 alkyl, C_1-C_6 alkenyl, C_4-C_6 aryl, halo, CF_3, OCH_3, NO_2, CN, OH, NMe_2, COOH, and $COOCH_3$; X is –CH- or N; R^2 is hydrogen (H), alkenyl ($=CH_2$), or oxo (=O); and dashed lines represent bonds that are independently present or absent.

[0032] In certain embodiments, compounds of Formula I, Ia, Ib, Ic and II are selected from the following compounds, and pharmaceutically acceptable salts thereof:

Table 1



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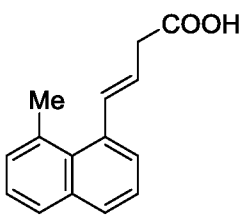

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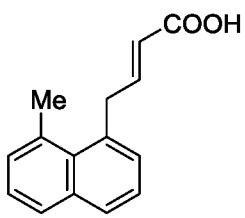

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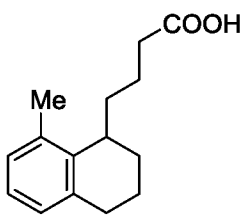

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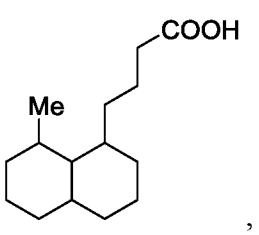
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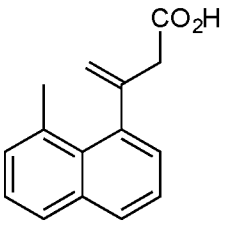
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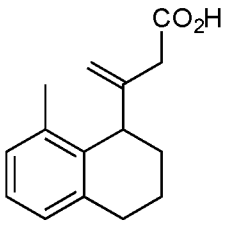
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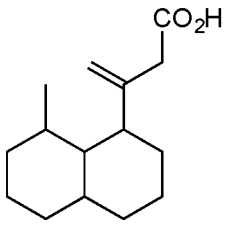
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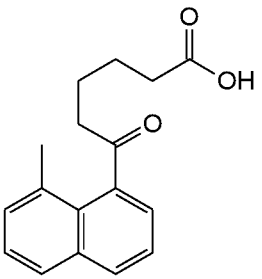
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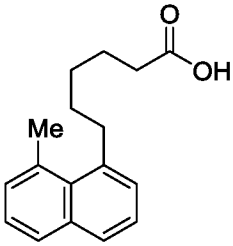
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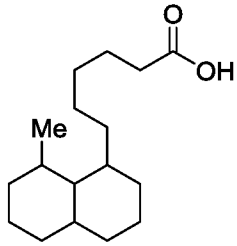
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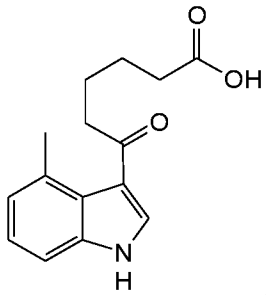
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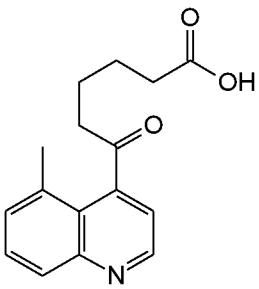
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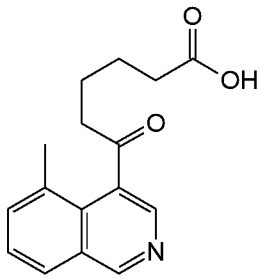
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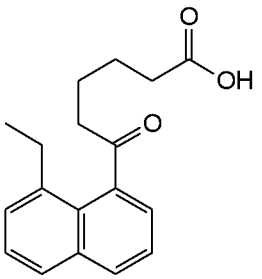
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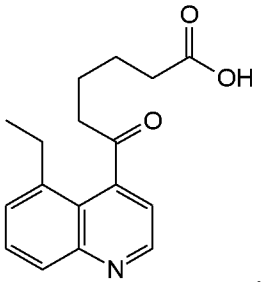
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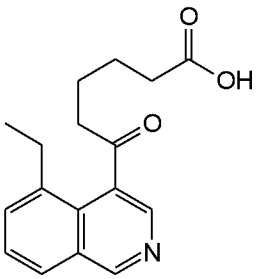
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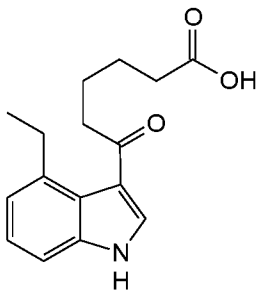
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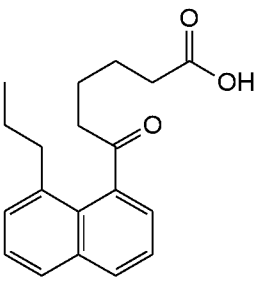
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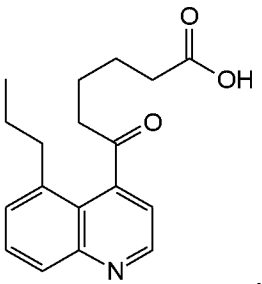
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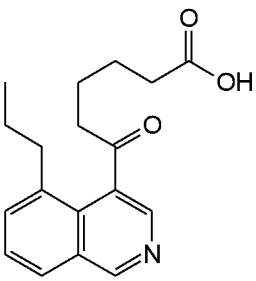
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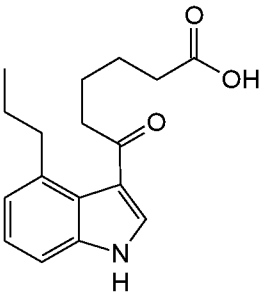
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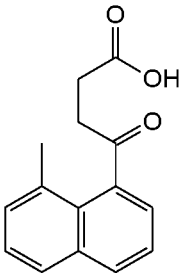
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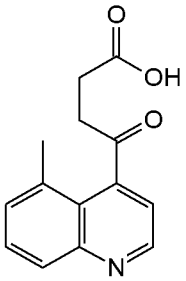
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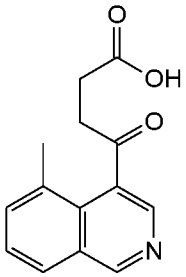
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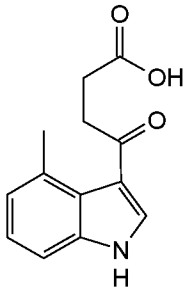
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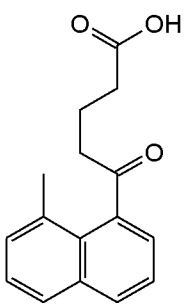
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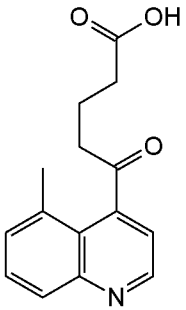
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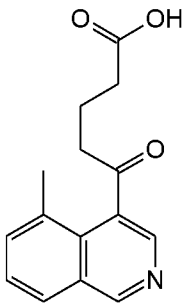
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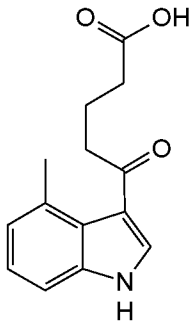
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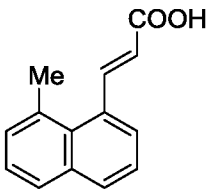
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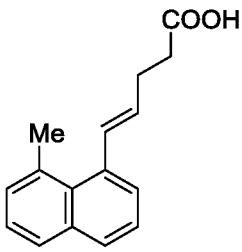
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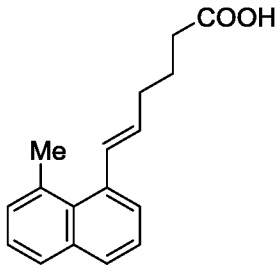
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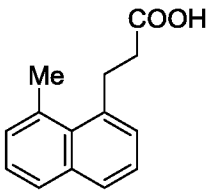
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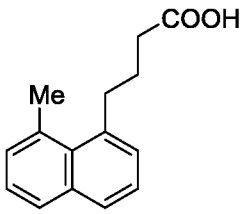
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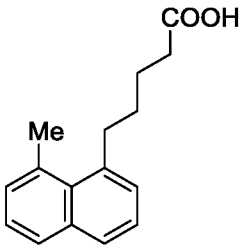
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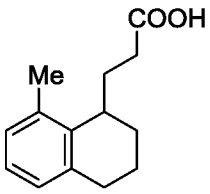
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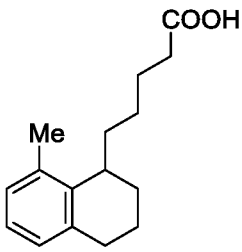
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5a 5c

[0033] In certain embodiments, compounds disclosed herein bind to the regulatory pocket of ToxT with a K_d of less than about 300 μM.

[0034] In another aspect, provided herein is a compound comprising a carboxylate moiety capable of interacting with Lys31 of the N-terminal domain and Lys230 of the C-terminal domain of the fatty acid binding region of ToxT, and a fused bicyclic moiety. In one embodiment, the fused bicyclic moiety is selected from the group consisting of naphthalene, 1,2,3,4-tetrahydronaphthalene, 1,2,3,4,5,6,7,8-octahydronaphthalene, decalin, isoquinoline, and quinolone. In another embodiment, the fused bicyclic moiety is capable of interacting with Phe22 of the fatty acid binding region of ToxT. In still another embodiment, the compound binds to the regulatory pocket of ToxT with a K_d of less than about 300 μM, as measured by a method known to those skilled in the art or described herein. In a particular embodiment, the compound displaces the natural (e.g., endogeneous) fatty acid ligand from the fatty acid binding region of ToxT, as measured by a method known to those skilled in the art or described herein. In another particular embodiment, the compound inhibits tcpA expression as determined by a β-galactosidase assay as described herein or another method known to those skilled in the art.

[0035] In another aspect, provided herein is a method for preventing, mitigating, or treating an infection by a bacterium that expresses an A/X regulatory protein comprising administering to a subject in need thereof an effective amount of a disclosed compound, or a pharmaceutical composition thereof, such that the infection is prevented, mitigated, or treated. In one embodiment, the method is for preventing an infection. In another embodiment, the method is for mitigating an infection. In another embodiment, the method is for treating an infection. In another embodiment, the bacterium is selected from the group consisting of *Vibrio cholerae, Escherichia coli, Shigella flexneri, Yersinia enterocolitica, Yersinia pestis, Brucella abortus, Salmonella typhi, Bacillus anthracis, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella typhimurium*.

[0036] In another aspect, provided herein is a method for decreasing expression of a bacterial virulence factor comprising contacting a bacterium that expresses an A/X regulatory protein with a disclosed compound, or a pharmaceutically acceptable salt thereof, such that the expression of a virulence factor by said bacterium is decreased. In

one embodiment, the bacterium is selected from the group consisting of *Vibrio cholerae, Escherichia coli, Shigella flexneri, Yersinia enterocolitica, Yersinia pestis, Brucella abortus, Salmonella typhi, Bacillus anthracis, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella typhimurium*.

Design of small molecule inhibitors

[0037] The X-ray structure of ToxT revealed a 16-carbon monounsaturated fatty acid cis-palmitoleate bound in a pocket within the N-terminal domain, the homologous location as the arabinose-binding site within AraC, a related protein for which the family is named. The fatty acid bridges the interface between the N-terminal dimerization domain and C-terminal DNA-binding domain of ToxT, suggesting a potential mechanism for fatty acid-mediated inhibition. The long aliphatic chain abuts the hydrophobic residues within the N-terminal domain and helices 9 and 10 of the C-terminal domain, occupying the bulk of the binding pocket (Fig. 3). The anionic carboxylate forms salt bridges with C-terminal and N-terminal lysine residues, presumably locking ToxT in a "closed" conformation in which it is unable to dimerize and/or bind DNA. Based on this model, a conformational change in the ligand-binding pocket and domain interface can easily impact the nature of the DNA-binding domain or the dimerization domain. Thus, it was hypothesized that the carboxylate head and hydrophobic tail responsible for inter-domain interactions is critical for inhibition, and maintaining fatty acid-like character is required for an effective *V. cholerae* anti-virulence drug. Using the bound conformation of cis-palmitoleate as a template, a subset of small molecule-inhibitors have been designed with these general characteristics (Figures 4A-B).

[0038] Additionally, compounds disclosed herein comprise a fused ring system that should provide rigidity and lead to tighter binding. In contrast to the natural ligand, which must assume a constrained fold in the binding pocket, the "pre-folded" bicyclic compounds disclosed herein have already overcome this entropic penalty. The initial subset of compounds includes variations in both the length of the carboxylate chain and degree of saturation of the ring system.

[0039] Inhibitors prevent virulence factor expression and ToxT-DNA binding

[0040] Initial screening of the synthetic inhibitors in a *V. cholerae* classical biotype transcriptional fusion system (*tcpA-lacZ*) revealed marked inhibition of *tcpA* expression,

as measured in a β-galactosidase reporter assay (Fig. 5A). All ten compounds inhibited tcpA transcription significantly more than virstatin, a molecule known to inhibit regulation of *V. cholerae* virulence (Shakhnovich, E. A., Hung, D. T., Pierson, E., Lee, K. & Mekalanos, J. J. Virstatin inhibits dimerization of the transcriptional activator ToxT. *Proceedings of the National Academy of Sciences* 104, 2372-2377 (2007)), whereas *cis*-palmitoleic and oleic acids had essentially no effect at these concentrations (Fig. 5A). At 5μM, the strongest inhibitors decreased *tcpA-lacZ* transcription levels to almost baseline (that of *ΔToxT tcpA-lacZ*), but did not affect the number of colony forming units (data not shown). Expression of TcpA by Western Blot provided further evidence for the potency of the inhibitors described herein, as the degree of inhibition of each compound was consistent with that determined by the β-galactosidase assay (Fig. 5B). Similarly, compounds 4a, 5a, 3b, and 4b completely abolished autoagglutination of O395 cultures at 0.5μM, while virstatin did not (Fig. 5C). When cells are grown under inducing conditions, the production of TCP pili allows for the formation of microcolonies, clusters of bacterial cells that are tethered together and which form a pellet in the bacterial culture. This process is dependent on production of the major pilin subunit TcpA. Pili formation was inhibited by compounds 4a, 5a, 3b, and 4b even at concentrations as low as 50 nM (Fig. 5C). As with the β-gal assay, cis-palmitoleic and oleic fatty acids had no affect at these concentrations (Figs. 9A-B).

[0041] It has been shown that virstatin and unsaturated fatty acids (UFAs) act on ToxT directly by affecting its ability to dimerize and/or bind DNA (Lowden, M. J. *et al.* Structure of Vibrio cholerae ToxT reveals a mechanism for fatty acid regulation of virulence genes. *Proceedings of the National Academy of Sciences* **107**, 2860-2865 (2010); Childers, B. M. *et al.* N-terminal Residues of the Vibrio cholerae Virulence Regulatory Protein ToxT Involved in Dimerization and Modulation by Fatty Acids. *Journal of Biological Chemistry* **286**, 28644-28655 (2011)). To determine if the compounds acted in a similar manner, electrophoretic mobility shift assays (EMSAs) were performed using purified ToxT protein and a digoxigenin-labeled 84 base-pair segment of the *tcpA* promoter. All compounds and virstatin prevented ToxT from binding DNA (Fig. 6A). The same concentration of ToxT did not shift a similar altered probe that ToxT is unable to bind due to a mutation in the binding site at the *tcpA* promoter (Hulbert, R. R. & Taylor, R. K. Mechanism of ToxT-Dependent Transcriptional

Activation at the Vibrio cholerae tcpA Promoter. *J. Bacteriol.* **184**, 5533-5544 (2002)) (Fig. 6B). The compounds with greatest inhibition of tcpA expression as determined by the β-galactosidase assay were assessed further in order to determine the concentration threshold required for inhibition. Compounds 4a, 5a, 3b, and 4b inhibited DNA-binding more strongly than virstatin, as seen in the dose-response EMSA (Fig. 6C).

X-ray crystal structures of ToxT-inhibitor complexes and computational modeling

[0042] While it is clear the above compounds are effective inhibitors of ToxT, in order to better understand the molecular basis of inhibition and elucidate the specific residues and interactions necessary for binding, the computational docking program AutoDock (Morris, G. M. *et al.* AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *Journal of computational chemistry* 30, 2785-2791 (2009)) was used to predict how the compounds bind to ToxT. No significant differences in the free energies of binding were predicted, although there were slight modifications to the orientation of the docked ligands (Fig. 10). In order to verify the structures of the AutoDock models, the crystal structures of ToxT bound to two of the leading compounds, 3b and 5a, were solved to 2.0 Å and 2.3 Å, respectively. Clear electron density was immediately visible in the fatty acid binding pocket of both structures, but neither could accommodate palmitoleic, oleic, or palmitic acids, confirming the inhibitors had bound in place of fatty acid (Figs. 7A-C, Fig. 11). The crystal structures of the inhibitors bound to ToxT were remarkably similar to those predicted by AutoDock, suggesting the computational models for compounds in which the X-ray structures were not solved are likely accurate. In the two crystal structures and all of the AutoDock structures, the compounds bound similarly to the fatty acid (Figs. 7B and 7C), with the position of the carboxylate varying only by 0.02-0.87 Å, suggesting favorable electrostatic interactions between the polar head group and Lys31 and Lys230. Other than the carboxylate, it seems that the bound conformation is dictated by the planarity of the compound. For the more saturated, more planar naphthalene compounds, the aliphatic ring system is rotated with respect to the fatty acid backbone. The tetralin rings, in contrast, bind further into the pocket, with the carbons in the carboxylate chain tracing the fatty acid almost perfectly (Fig. 7B, Fig. 10).

Inhibitors bind ToxT more tightly than virstatin

[0043] Saturation transfer difference (STD) NMR was used to characterize the strength of ToxT-ligand binding interactions. Significant STD signal was observed for virstatin, and confirms it is binding to ToxT. As shown in Fig. 8A, saturation is transferred to the aromatic protons of virstatin upon binding the protein. The STD effect on the aliphatic protons of virstatin was not analyzed due to their spectral overlap with ToxT. Based on the STD spectra for the aromatic protons $H_{a,b}$ and $H_{e,f}$, the K_d of virstatin was determined to be 483±109 μM and 331±65 μM respectively (Fig. 12). The relative binding affinities of compounds 3b and 5a were determined by competition STD NMR experiments. The compounds were titrated into samples containing 20 μM ToxT and 100 μM virstatin, causing a significant decrease in the STD signals of virstatin (Fig. 8B,C). As X-ray crystallography has confirmed the binding pocket of compounds 3b and 5a, and because the STD signal of virstatin decreased upon the addition of the competitors, it is evident that virstatin also binds in the same regulatory pocket of ToxT. Based on the calculated K_d of virstatin and the decrease in the STD signal, the calculated K_i values of 3b and 5a were 10 μM and 31 μM, respectively. Despite a suggested tendency for virstatin to bind non-specifically at high concentrations, the relative magnitudes of these binding constants are consistent with the activity assays described above.

Discussion

[0044] Compounds disclosed herein are inhibitors of ToxT, the master regulator of virulence for the disease cholera. These inhibitors were designed based on the conformation of the ToxT ligand found in the X-ray crystal structure, *cis*-palmitoleic acid (Lowden, M. J. *et al.* Structure of Vibrio cholerae ToxT reveals a mechanism for fatty acid regulation of virulence genes. *Proceedings of the National Academy of Sciences* **107**, 2860-2865 (2010)). These "pre-folded" small molecules bind much more tightly than unsaturated fatty acids, which show no activity at the tested concentrations. The compounds do not inhibit colony formation, and therefore are not bactericidal, but were shown to inhibit virulence gene production via β-galactosidase and autoagglutination assays, and ToxT-DNA binding via EMSA. The compounds bind to the ToxT regulatory domain pocket as predicted by AutoDock and visualized by X-ray crystallography. Based on a calculated K_d determined by STD NMR, the lead compounds have at least 10-fold stronger binding affinities than the best-known ToxT inhibitor, virstatin (Shakhnovich, E. A., Hung, D. T., Pierson, E., Lee, K. & Mekalanos,

J. J. Virstatin inhibits dimerization of the transcriptional activator ToxT. *Proceedings of the National Academy of Sciences* **104**, 2372-2377 (2007); Hung, D. T., Shakhnovich, E. A., Pierson, E. & Mekalanos, J. J. Small-Molecule Inhibitor of Vibrio cholerae Virulence and Intestinal Colonization. *Science* **310**, 670-674 (2005)).

[0045] The STD NMR data also indicate that virstatin binds in the same ligand-binding pocket of ToxT, which seems to accommodate only one ligand at a time. Similarly, as further verified in the X-ray crystal structures of the ToxT-inhibitor complexes, compounds disclosed herein displace the fatty acid and bind in its place.

Definitions

[0046] As used herein, the term "alkyl" refers to a straight or branched chain hydrocarbon, preferably having from one to six carbon atoms (*i.e.*, C_1-C_6). Examples of "alkyl" as used herein include methyl, ethyl, propyl, isopropyl, n-butyl, isopentyl, n-pentyl, and the like, as well as substituted versions thereof. In particular embodiments, an alkyl of the invention is a C_1-C_3 alkyl.

[0047] The term "alkylidenyl" refers to a divalent functional group derived from an alkane by removal of two hydrogen atoms from the same carbon atom, the free valencies being part of a double bond. For example, a methylidene substituent has the formula $=CH_2$.

[0048] The term "alkenyl" refers to a straight or branched chain aliphatic hydrocarbon containing one or more carbon-to-carbon double bonds that may be optionally substituted, with multiple degrees of substitution included within the present invention. Examples include, but are not limited to, vinyl, allyl, and the like, as well as substituted versions thereof.

[0049] The term "aryl" refers to a monovalent group with an aromatic carbon atom as the point of attachment, said carbon atom forming part of a four-, five- or six-membered aromatic ring structure wherein the ring atoms are all carbon, and wherein the monovalent group is composed of carbon and hydrogen. Non-limiting examples of aryl groups include phenyl, methylphenyl, (dimethyl)phenyl, -ethylphenyl, propylphenyl, -$C_6H_4CH(CH_3)_2$, -$C_6H_4CH(CH_2)_2$, methylethylphenyl, vinylphenyl, naphthyl, and the monovalent group derived from biphenyl. In particular embodiments, the aryl is a phenyl group.

[0050] Any undefined valency on an atom of a structure shown in this application implicitly represents a hydrogen atom bonded to the atom.

[0051] The term "hydrate" when used as a modifier to a compound means that the compound has less than one (*e.g.*, hemihydrate), one (*e.g.*, monohydrate), or more than one (*e.g.*, dihydrate) water molecules associated with each compound molecule, such as in solid forms of the compound.

[0052] An "isomer" of a first compound is a separate compound in which each molecule contains the same constituent atoms as the first compound, but where the configuration of those atoms in three dimensions differs.

[0053] A "pharmaceutically acceptable salt" refers to a salt of a disclosed compound which is pharmaceutically acceptable, and e.g., which possesses the desired pharmacological activity. Such salts include acid addition salts formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, and the like; or with organic acids such as 1,2-ethanedisulfonic acid, 2-hydroxyethanesulfonic acid, 2-naphthalenesulfonic acid, 3-phenylpropionic acid, 4,4'-methylenebis(3-hydroxy-2-ene-1-carboxylic acid), 4-methylbicyclo[2.2.2]oct-2-ene-1-carboxylic acid, acetic acid, aliphatic mono- and di-carboxylic acids, aliphatic sulfuric acids, aromatic sulfuric acids, benzenesulfonic acid, benzoic acid, camphorsulfonic acid, carbonic acid, cinnamic acid, citric acid, cyclopentanepropionic acid, ethanesulfonic acid, fumaric acid, glucoheptonic acid, gluconic acid, glutamic acid, glycolic acid, heptanoic acid, hexanoic acid, hydroxynaphthoic acid, lactic acid, laurylsulfuric acid, maleic acid, malic acid, malonic acid, mandelic acid, methanesulfonic acid, muconic acid, *o*-(4-hydroxybenzoyl)benzoic acid, oxalic acid, *p*-chlorobenzenesulfonic acid, phenyl-substituted alkanoic acids, propionic acid, *p*-toluenesulfonic acid, pyruvic acid, salicylic acid, stearic acid, succinic acid, tartaric acid, tertiarybutylacetic acid, trimethylacetic acid, and the like. Pharmaceutically acceptable salts also include base addition salts which may be formed when acidic protons present are capable of reacting with inorganic or organic bases. Acceptable inorganic bases include sodium hydroxide, sodium carbonate, potassium hydroxide, aluminum hydroxide and calcium hydroxide. Acceptable organic bases include ethanolamine, diethanolamine, triethanolamine, tromethamine, *N*-methylglucamine and the like. It should be recognized that the

particular anion or cation forming a part of any salt of this invention is not critical, so long as the salt, as a whole, is pharmacologically acceptable. Additional examples of pharmaceutically acceptable salts and their methods of preparation and use are presented in *Handbook of Pharmaceutical Salts: Properties, and Use* (P. H. Stahl & C. G. Wermuth eds., Verlag Helvetica Chimica Acta, 2002).

[0054] Disclosed compounds may also exist in prodrug form. Since prodrugs are known to enhance numerous desirable qualities of pharmaceuticals, *e.g.*, solubility, bioavailability, manufacturing, etc., the compounds employed in some methods of the invention may, if desired, be delivered in prodrug form. Thus, the invention contemplates prodrugs of compounds of the present invention as well as methods of delivering prodrugs. Prodrugs of the compounds employed in the invention may be prepared by modifying functional groups present in the compound in such a way that the modifications are cleaved, either in routine manipulation or *in vivo*, to the parent compound. Accordingly, prodrugs include, for example, compounds described herein in which a hydroxy or carboxy group is bonded to any group that, when the prodrug is administered to a patient, cleaves to form a hydroxy or carboxylic acid, respectively. For example, a compound comprising a hydroxy group may be administered as an ester that is converted by hydrolysis *in vivo* to the hydroxy compound. Suitable esters that may be converted *in vivo* into hydroxy compounds include acetates, citrates, lactates, phosphates, tartrates, malonates, oxalates, salicylates, propionates, succinates, fumarates, maleates, methylene-bis-β-hydroxynaphthoate, gentisates, isethionates, di-*p*-toluoyltartrates, methanesulfonates, ethanesulfonates, benzenesulfonates, *p*-toluenesulfonates, cyclohexylsulfamates, quinates, esters of amino acids, and the like.

[0055] Disclosed compounds can be prepared as described herein (see Example 1) or using any suitable methodology routinely practiced in the art, and be analyzed for their pharmacological properties by routine methodologies. For example, kinetic solubility can be measured using a direct UV absorbance method or thermodynamic solubility can be measured. In addition, stability in gastrointestinal fluids can be determined by conventional methods (Asafu-Adjaye, et al. (2007) *J. Pharm. Biomed. Anal.* 43:1854-1859), *e.g.*, 1 hour in simulated gastric fluid (pH 1.2, pepsin) at 37°C and/or 3 hours in simulated intestinal fluid (pH 6.8, pancreatin). Furthermore, using the Parallel Artificial Membrane Permeability Assay (PAMPA)-blood-brain barrier (BBB) permeability assay

(Di, et al. (2009) *J. Pharm. Sci.* 98:1980-1991) or B-P dialysis (Kalvass & Maurer (2002) *Biopharm. Drug Dispos.* 23(8):327-38), brain penetration can be assessed. Furthermore, lipophilicity can be estimated by partitioning between octanol and water using a shake flask method or pH metric method and permeability can be assessed using the Caco-2 cell layer method of PAMPA assay.

[0056] A disclosed compound may be administered in a pharmaceutical composition by various routes including, but not limited to, intradermal, intramuscular, intraperitoneal (*e.g.,* by injection), intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intracerebral, transdermal, rectal, or topical administration. Depending on the route of administration, the active compound may be coated. For example, to administer the therapeutic compound by a route other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. By way of illustration, the therapeutic compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan, et al. (1984) *J. Neuroimmunol.* 7:27). Accordingly, provided herein is a pharmaceutical composition comprising a compound disclosed herein and a pharmaceutically acceptable carrier.

[0057] When the compound is to be administered parenterally, intraperitoneally, intraspinally, or intracerebrally, dispersions can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

[0058] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol,

polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, sodium chloride, or polyalcohols such as mannitol and sorbitol, in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

[0059] Sterile injectable solutions can be prepared by incorporating the therapeutic compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the therapeutic compound into a sterile carrier which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (*i.e.,* the therapeutic compound) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0060] A disclosed compound can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The therapeutic compound and other ingredients may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the therapeutic compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The percentage of the therapeutic compound in the compositions and preparations may, of course, be varied. The amount of the therapeutic compound in such therapeutically useful compositions is such that a suitable dosage will be obtained.

[0061] It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used

herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit containing a predetermined quantity of therapeutic compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the therapeutic compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such a therapeutic compound for the treatment of a selected condition in a subject.

[0062] In addition to conventional pharmaceutical compositions, this invention also provides nutraceuticals; nutritional compositions, such as dietary supplements; medical nutrition or functional foods including a compound of Formula I. Such compositions can be prepared by mixing one or more compounds of the invention with an edible nutritionally acceptable solid or liquid carriers and/or excipients, *e.g.,* fillers, such as cellulose, lactose, sucrose, mannitol, sorbitol, and calcium phosphates; and binders, such as starch, gelatin, tragacanth, methylcellulose and/or polyvinylpyrrolidone (PVP). Optional additives include lubricants and flow conditioners, *e.g.,* silicic acid, silicon dioxide, talc, stearic acid, magnesium/calcium stearates and polyethylene glycol (PEG) diluents; disintegrating agents, *e.g.,* starch, carboxymethyl starch, cross-linked PVP, agar, alginic acid and alginates, coloring agents, flavoring agents and melting agents. The composition of the invention can optionally include conventional food additives, such as emulsifiers, stabilizers, sweeteners, preservatives, chelating agents, osmotic agents, buffers or agents for pH adjustment, acidulants, thickeners, texturizers and the like.

[0063] In addition to the above, the compositions of the present invention can further include antibiotics (*e.g.*, tetracyclines), probiotics, prebiotics, anti-LPS sIgA (Apter, et al. (1993) *Infect. Immun.* 61(12):5279–5285), as well as other monounsaturated fatty acids such as oleic acid or palmitoleic acid to facilitate the prevention, mitigation and/or treatment of a bacterial infection. As such, compositions containing other monounsaturated fatty acids such as oleic acid, palmitoleic acid and vaccenic acid and their use in the treatment of bacterial infections are also embraced by the present invention.

[0064] Suitable product formulations according to the present invention include sachets, soft gel, powders, syrups, pills, capsules, tablets, liquid drops, sublinguals, patches, suppositories, liquids, injectables and the like. Also contemplated are food and beverage products containing one or more compounds of the present invention, such as solid food products, like bars (*e.g.,* nutritional bars or cereal bars), powdered drinks, dairy products, breakfast cereals, muesli, candies, confectioneries, cookies, biscuits, crackers, chocolate, chewing-gum, desserts and the like; liquid comestibles, like soft drinks, juice, sports drinks, milk drinks, milk-shakes, yogurt drinks or soups, etc. The addition of one or more compounds of the invention to animal feed is also included within the scope of this invention.

[0065] The disclosed compositions can be provided as a component of a meal, *e.g.*, a nutritional or dietary supplement, in the form of a health drink, a snack or a nutritionally fortified beverage, as well as a conventional pharmaceutical, *e.g.,* a pill, a tablet or a softgel, for example.

[0066] *ToxT.* ToxT belongs to the AraC/XylS (A/X) superfamily of regulatory proteins. This family is composed of approximately 1,974 members identified in 149 bacterial genomes including *Bacillus anthracis, Listeria monocytogenes*i and *Staphylococcus aureus* (Ibarra, et al. (2008) *Genetica* 133:65-76), and is known for its role in virulence gene regulation. The crystal structure of ToxT identified a binding pocket enclosed by residues Y12, Y20, F22, L25, I27, K31, F33, L61, F69, L71, V81, and V83 from the N-terminal domain and residues I226, K230, M259, V261, Y266, and M269 from the C-terminal domain. The volume of this predominantly hydrophobic pocket is 780.9 $Å^3$ as calculated by the program CASTp. This pocket contains a sixteen-carbon fatty acid with a negatively charged carboxylate head group forming salt bridges with both K31 from the N-terminal domain and K230 from the C-terminal domain. Using secondary structure prediction and homology modeling, multiple candidates from the A/X protein superfamily were identified, which contained lysines or other positive amino acids at positions homologous to those identified in ToxT. This analysis indicated that A/X regulatory proteins from many pathogenic bacteria, including *Vibrio cholerae, Escherichia coli, Shigella flexneri, Yersinia enterocolitica, Yersinia pestis, Brucella abortus, Salmonella typhi, Bacillus anthracis,* and *Clostridium botulinum* (Figure 1), as well as *Staphylococcus aureus* and *Salmonella typhimurium* contain with homologous

lysine residues and/or homologous ligand binding pockets. Thus, use of compositions herein can be broadly applied to treat enteric bacterial infections that cause travelers' diarrhea, salmonella, brucellosis, botulism, dysentery, and typhoid fever, diseases infecting some 4 billion people annually worldwide.

[0067] Thus, the present invention embraces compositions containing one or more compounds of the invention for use in methods for decreasing or inhibiting the expression of bacterial virulence genes. This method is carried out by contacting a pathogenic bacterium with a composition of the present invention so that the expression of at least one virulence factor, *e.g.,* TCP and/or CT in *V. cholerae*, is measurably decreased as compared to bacteria not contacted with the composition of the invention. A decrease or inhibition of virulence factor expression can be measured using any conventional method for monitoring nucleic acid or protein levels in a cell, *e.g.,* northern blot analysis, RT-PCR analysis, dot blot analysis, western blot analysis and the like. Desirably, the composition of the invention decreases virulence factor expression by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or as much as 100% as compared to untreated bacteria.

[0068] *V. cholerae.* There are several characteristics of pathogenic *V. cholerae* that are important determinants of the colonization process. These include adhesins, neuraminidase, motility, chemotaxis and toxin production. If the bacteria are able to survive the gastric secretions and low pH of the stomach, they are well adapted to survival in the small intestine. *V. cholerae* is resistant to bile salts and can penetrate the mucus layer of the small intestine, possibly aided by secretion of neuraminidase and proteases. Specific adherence of *V. cholerae* to the intestinal mucosa is likely mediated by the long filamentous TCP pili which are coregulated with expression of the cholera toxin genes.

[0069] As indicated herein, *V. cholerae* produces cholera toxin, which is composed of two A subunits and five B subunits. The B subunits allow binding to a ganglioside (GM_1) receptor on the intestinal epithelial cells. The B pentamer must bind to five corresponding GM_1 receptors. This binding occurs on lipid rafts, which anchor the toxin to the membrane for endocytosis of the A subunits, thereby trafficking the toxin into the cell and to the basolateral surface where it acts (Lencer (2001) *Am. J. Physiol.*

Gastrointest. Liver Physiol. 280:G781-G786). Once internalized, the A subunits proteolytically cleave into A1 and A2 peptides. The A1 peptide ADP-ribosylates a GTP-binding protein, thereby preventing its inactivation. The always active G protein causes adenylate cyclase to continue forming cAMP. This increase in intracellular cAMP blocks absorption of sodium and chloride by microvilli and promotes the secretion of water from the intestinal crypt cells to preserve osmotic balance (Torgersen, et al. (2001) *J. Cell Sci.* 114:3737-3747). This water secretion causes the watery diarrhea with electrolyte concentrations isotonic to plasma. The fluid loss occurs in the duodenum and upper jejunum, with the ileum less affected. The colon is less sensitive to the toxin, and is therefore still able to absorb some fluid. The large volume, however, overwhelms the colon's absorptive capacity.

[0070] In addition to *V. cholerae,* the following is a list of some of the bacterial enteric pathogens that express A/X family members that properly align with ToxT. In so far as other pathogens may be identified based upon the structural analysis disclosed herein, the following list is merely illustrative and in no way limits the scope of bacteria that can be targeted by the instant fatty acid compositions.

[0071] *Escherichia coli*. There are several pathogenic derivatives of *E. coli*. Several of the most common are as follows. One is Enterohemorrhagic *E. coli* (EHEC), which causes a Shigella-like illness and is also known as the hamburger meat *E. coli*. Another is Enteropathogenic *E. coli* (EPEC), which causes persistent diarrhea in children. EPEC expresses a surface appendage termed the bundle forming pilus, or BFP. BFP is required for intestinal colonization by the bacterium. BFP gene expression is activated by the A/X family member PerA that meets alignment criteria described herein. A third example is Enterotoxigenic *E. coli* (ETEC), which expresses a toxin identical to ToxT and causes traveler's diarrhea. ETEC expresses colonization factor adhesions termed CS1 and CS2. The expression of the corresponding genes is activated by an A/X family regulator termed Rns that meets alignment criteria described herein. Similarly, the *cof* gene cluster, *Longus* gene cluster and CFA/I operon of ETEC also respectively encode regulatory proteins cofS, lngS and CfaD, which regulate the expression of virulence factors. Indeed, CfaD and Rns are fully interchangeable with each other (Bodero, et al. (2007) *J. Bacteriol.* 189:1627-32) and recognize the same DNA binding sites.

[0072] *Salmonella.* Salmonella cause 1.4 million cases of gastroenteritis and enteric fever per year in the US and lead all other food borne pathogens as a cause of death. While there are over a thousand serotypes of Salmonella that can cause gastroenteritis, *S. enteritidis* (sv. Typhimurium) is the leading cause. *S. enteritidis* (sv. Typhimurium) infection of mice serves as a model for typhoid fever as the causative agent of this disease only infects humans. As such, this species has served as a model organism for both gastroenteritis and typhoid fever. Most of the genes that encode virulence factors are located in clusters on salmonella pathogenicity islands termed SPIs. SPI-1 carries the genes for a type III secretion system (T3SS), the expression of which is critical for virulence. The master regulator of the expression of SPI-1 genes is HilA. The expression of HilA itself is controlled by HilD. HilD is an A/X family member that meets alignment criteria described herein.

[0073] *Salmonella typhi* (*S. enterica* sv. Typhi) is the leading cause of enteric fever also known as typhoid fever. Typhoid fever is estimated to affect approximately 17 million people annually, causing 600,000 deaths. *S. typhi* is a multi-organ organism, infecting lymphatic tissues, liver, spleen, and bloodstream. *S. typhi* has a gene regulatory network similar to the SPI-1 and regulation of T3SS gene expression in *S. enteritidis* (sv. Typhimurium). In the case of *S. typhi* the aligned A/X family member is designated SirC.

[0074] *Shigella.* Several Shigella species are responsible for the majority of bacillary dysentery that is caused by this organism. *S. dysenteriae* is common in many parts of the world. *S. flexneri* and *S. sonnei* are the most common in the U.S. Most molecular analysis regarding Shigella has been performed with *S. flexneri*. This species requires a surface protein, IcsA, to nucleate actin and travel through and between host cells. Expression of the *icsA* gene is activated by VirF, which meets alignment criteria described herein.

[0075] *Bacillus anthracis*. *Bacillus anthracis* is an aerobic spore-forming bacteria that causes anthrax disease. Livestock may become infected by eating or inhaling anthrax spores. Humans, especially farmers and individuals who work in slaughterhouses, may develop cutaneous anthrax through skin exposure to infected animals. Humans can also get inhalational anthrax by breathing in material contaminated with the bacteria. This bacterium also expresses an AraC family member.

[0076] *Listeria. Listeria monocytogenes* is a facultative intracellular bacterium that is the causative agent of Listeriosis. It is one of the most virulent food-borne pathogens with 20 to 30 percent of clinical infections resulting in death. *Listeria monocytogenes* also expresses an AraC family member.

[0077] *Staphylococcus aureus. Staphylococcus aureus* is a facultatively anaerobic, gram-positive coccus and is the most common cause of staph infections. Some strains of *S. aureus*, which produce the exotoxin TSST-1, are the causative agents of toxic shock syndrome, whereas other strains of *S. aureus* also produce an enterotoxin that is the causative agent of *S. aureus* gastroenteritis.

[0078] *Yersinia enterocolitica* is a common pathogen of children and adults, with a strong propensity for extraintestinal complications. Gastrointestinal disorders include enterocolitis, particularly in children, and pseudoappendicitis, particularly in young adults. *Y. enterocolitica* virulence factors include outer proteins termed Yops and YadA, which is an adhesin that is essential for colonization. VirF is an A/X family member that meets alignment criteria described herein.

[0079] *Yersinia pestis* is the pathogen of human and animals that causes all three main forms of the plague including pneumonic, septicemic and bubonic plagues. Similar to VirF, the *Y. pestis* virulence regulon is controlled by the A/X family member LcrF (Hoe, et al. (1992) *J. Bacteriol.* 174:4275-86).

[0080] In addition, *Bacillus anthracis,* the etiologic agent of anthrax; Brucella abortus, which causes brucellosis; and *Clostridium botulinum*, the causal agent of botulism, each have an A/X family member that meets alignment criteria described herein.

[0081] In so far as ToxT and other A/X regulatory proteins directly regulate the expression of virulence factors, which are involved in pathogenicity, inhibition of A/X regulatory protein activity, and hence virulence factor expression, is useful in the prevention, mitigation, and/or treatment of enteropathogenic bacterial infection. As used herein, the term “bacterial infection” is used to describe the process of adherence and virulence factor production by a pathogenic bacterium that expresses an A/X regulatory protein. For the purposes of the present invention, the term “treatment” or “treating” means any therapeutic intervention in a mammal, preferably a human or any other animal

suffering from a enteropathogenic bacterial infection, such that symptoms and bacterial numbers are reduced or eliminated. By way of illustration, it is contemplated that by reducing adhesion of *V. cholerae* to the intestinal mucosa via TCP pili, colonization will be reduced or inhibited, thereby allowing the subject to clear the bacterial infection.

[0082] "Prevention" or "preventing" refers to prophylactic treatment, wherein clinical symptom development is delayed or inhibited, *e.g.,* preventing infection from occurring and/or developing to a harmful state.

[0083] "Mitigation" or "mitigating" means arresting the development of clinical symptoms, *e.g.,* stopping an ongoing infection to the degree that it is no longer harmful, or providing relief or regression of clinical symptoms, *e.g.,* a decrease in fluid loss resulting from an infection.

[0084] Prophylactic or therapeutic treatment involves the administration of an effective amount of a compound of this invention to a subject in need thereof, thereby preventing, mitigating, or treating a bacterial infection. Subjects benefiting from the method of the invention include those having a bacterial infection (*e.g.,* exhibiting signs or symptoms) or those at risk of having a bacterial infection (*e.g.,* a subject exposed to a contaminated food or water source).

[0085] The terms "effective amount" means a dosage sufficient to measurably decrease or inhibit virulence gene expression and provide prevention, mitigation and/or treatment of a bacterial infection. In prophylactic or therapeutic applications, preferably the administered dose delays, mitigates, or reduces the signs and/or symptoms of infection in the subject by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The efficacy of a compound can be evaluated in an animal model system that may be predictive of efficacy in preventing, mitigating or treating the disease in humans.

[0086] The amount and dosage regimen of the composition of the invention to be administered is determined in the light of various relevant factors including the purpose of administration (*e.g.,* prevention, mitigation or treatment), the age, sex and body weight of an individual subject, and/or the severity of the subject's symptoms. In this respect, the

compositions of the invention can be administered under the supervision of a medical specialist, or may be self-administered.

[0087] Daily dosage of a composition of the present invention would usually be single or multiple servings per day, *e.g.*, once or twice daily, for acute or chronic use. However, benefit may be derived from dosing regimens that can include consumption on a daily, weekly or monthly basis or any combination thereof. Administration of compositions of the invention, *e.g.*, treatment, could continue over a period of days, weeks, months or years, until an infection has been treated. Optimally, the composition of the invention is consumed at least once a day on a regular basis, to prevent an infection.

[0088] The invention is described in greater detail by the following non-limiting examples.

Materials and Methods

β-galactosidase Assays

[0089] Cultures of the *V. cholerae* O395 *tcpA-lacZ* (MBN135) and *ΔToxT tcpA-lacZ* (MBN142) (Nye, M. B., Pfau, J. D., Skorupski, K. & Taylor, R. K. Vibrio cholerae H-NS Silences Virulence Gene Expression at Multiple Steps in the ToxR Regulatory Cascade. *Journal of Bacteriology* **182**, 4295-4303 (2000)) fusion constructs were grown for 14 hours with shaking in inducing conditions (LB media pH 6.5 at 30°C). For testing inhibitors, the compounds or DMSO were added to a final concentration of 0.05-50 μM at the time cultures were inoculated. β-galactosidase activity was quantitatively measured according to Miller (Miller, J. H. in *Experiments in molecular genetics* (Cold Spring Harbor Laboratory, 1972)).

Western Blot and Colony Formation Units Assay

Cell extracts from cultures grown as described above were subjected to 16% SDS-PAGE gels (Invitrogen), transferred to a nitrocellulose membrane, probed with anti-TcpA antibody, and visualized using the ECL detection system (Amersham). Serial dilutions of cell extracts grown in the presence or absence of the compounds were also plated on LB-

agar plates and CFUs were counted after overnight incubation. CFUs of cultures grown in the presence of compounds were compared to that of the wild-type O395 culture.

Autoagglutination Assays

[0090] Cultures of the *V. cholerae* classical strain O395 and O395*ΔtcpA* were grown for 14 hours with shaking in inducing conditions (LB media pH 6.5 at 30°C). The compounds or DMSO were added at the time cultures were inoculated. After 14 hours, cultures were placed at room temperature and observed immediately.

ToxT Expression and Purification

[0091] ToxT was expressed by autoinduction from *toxT-intein/CBD* (chitin binding domain) fusion construct transformed in BL21-CodonPlus (DE3)-RIL *E. coli*, as done previously (Lowden, M. J. *et al.* Structure of Vibrio cholerae ToxT reveals a mechanism for fatty acid regulation of virulence genes. *Proceedings of the National Academy of Sciences* **107**, 2860-2865 (2010)). Cells were harvested by centrifugation, resuspended in medium-salt column buffer (20 mM Tris, 1 mM EDTA, 500 mM NaCl, pH 8), lysed via sonication, and clarified by centrifugation. Clarified supernatant was loaded onto a gravity flow column packed with chitin beads (New England Biolabs) equilibrated in column buffer. After elution of the supernatant, the column was washed with column buffer followed by low-salt buffer (20 mM Tris, 1 mM EDTA, 200 mM NaCl, pH 8), and equilibrated with cleavage buffer (low-salt buffer with 100 mM dithiothreitol (DTT)). The column was placed at 4 °C for 16 hours to cleave the intein with the CBD. ToxT-intein/CBD fusion protein that co-eluted with the cleaved ToxT was separated using a HiTrap sepharose packed fast flow cationic exchange column (GE) with the following gradient: 45% high-salt buffer (20 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8) for 175 minutes at a flow rate of 0.4 ml/min.

Electrophoretic Mobility Shift Assays

[0092] An 84-bp *tcpA* promoter fragment was amplified from *V. cholerae* O395 chromosomal DNA by PCR using 5' labeled digoxigenin (DIG) primers: DIG-TCP-5 (5' TGTTTCTTTCA ATGCAAGTG) and DIG-TCP-6 (5' CACAAAGTCACCTACAATTG). Purified ToxT protein was mixed with 0.5 ng DIG-DNA in a binding buffer (10 mM Tris pH 7.5, 1 mM EDTA, 100 mM KCl, 5 mM

$MgCl_2$, 1 mM DTT, 0.3 mg/ml BSA, 0.25 μg poly [d(I-C)], and 10% glycerol). Compounds in DMSO were added to a final concentration of 0.1-100 μM, using the same volume of DMSO as a control. To show specificity, specific unlabeled dsDNA (84-bp *tcpA* promoter fragment) and non-specific labeled dsDNA (mutated *tcpA* promoter fragment CJ2.6) were added in excess. Reactions were incubated for 15 min at 30°C, loaded on a 5% polyacrylamide gel (1x Tris-Borate EDTA pH 8), and subjected to electrophoresis in 0.75x TBE at 4°C. The DNA was transferred onto a positively charged nylon membrane (Roche) by electroblotting using 0.5x TBE at 4°C, probed with anti-DIG-AP antibody, and visualized by chemiluminescence (Roche).

Crystallization

[0093] ToxT was co-crystallized with the synthesized compounds in hanging drops containing 50% protein buffer (20 mM Tris, 1 mM EDTA, ~300 mM NaCl, pH 7.5) and 50% reservoir solution (0.1 M MES pH 6.5 and 15% (w/v) PEG 400). The compound was added to 1.47 mg/ml ToxT at a 20:1 molar excess, and the complex was incubated at 30 °C for 15 minutes before setting up drops. The cryoprotectant for ToxT crystals contained 0.1 M MES pH 6.5, 18% (w/v) PEG 400, and either 30% 1,4-butanediol or glycerol.

X-ray data collection, structure solution, and refinement

[0094] Data sets were collected remotely at beam line GM/CA-XSD 23-ID-B at the Advanced Light Source at Argonne National Laboratory. Diffraction data were indexed and integrated with X-ray Detector Software (XDS) (Kabsch, W. XDS. *Acta Crystallographica* D66, 125-132 (2010)) and merged using Phenix(Adams, P. D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallographica* D66, 213-221 (2010)). Molecular replacement solutions were obtained with Phenix Phaser-MR (McCoy, A. J. *et al.* Phaser crystallographic software. *Journal of Applied Crystallography* 40, 658-674 (2007)) using ToxT (PDB ID: 3GBG) with PAM deleted from the PDB. The initial model was built via Phenix AutoBuild (Terwilliger, T. C. *et al.* Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard. *Acta Crystallographica* D64, 61-69 (2008)). Ligands were built from their SMILES strings using Phenix eLBOW (Moriarty, N. W., Grosse-Kunstleve, R. W. & Adams, P. D. electronic Ligand Builder and

Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallographica* D65, 1074-1080 (2009)). The corresponding CIF files were viewed and edited in Phenix REEL (Moriarty, et al., above). Ligands were manually placed into the electron density using Coot (Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallographica* D66, 486-501 (2010); Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallographica* D60, 2126-2132 (2004)). Iterative model refinement was performed using Phenix and Coot. Molecules of MES buffer were positioned in a similar manner as the ligands.

Molecular Docking

[0095] Computational screening was carried out using Autodock 4.0.1 (Morris, G. M. *et al.* AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *Journal of computational chemistry* 30, 2785-2791 (2009)). The coordinates for the receptor were obtained from the Protein Data Bank (PDB ID: 3GBG) and were modified to exclude the bound PAM. The ligands were prepared from their SMILES description in Chimera (Pettersen, E. F. *et al.* UCSF Chimera- A visualization system for exploratory research and analysis. *Journal of Computational Chemistry* 25, 1605-1612 (2004)). All ligands as well as CD_CE and CE_NZ of Lys230 and Lys31 of the receptor were considered flexible. A grid box with 35 X 40 X 32 points and a grid point spacing of 0.375 Å (gridcenter 54.5 X 46.5 X 20) encompassed the known binding pocket. Each docking simulation involved 20 evaluations using the Lamarkian genetic algorithm.

STD-NMR

[0096] Sample preparation: Virstatin was solubilized in DMSO-d_6 and compounds 5a and 3b in DMSO. Pure ToxT in protein buffer (20 mM Tris, 1 mM EDTA, ~300 mM NaCl, pH 8) was used at a final concentration of 20 μM. All samples contained 50 μM TSP as an internal standard and 5% D_2O. All NMR experiments were carried out on a Bruker Avance 600 MHz or 700 MHz spectrometer equipped with a TCI cryogenic probe. Samples were stored at 4 °C prior to acquisition.

[0097] Determination of K_d of virstatin: For on-resonance spectra, the protein was saturated at 500 Hz or 583.33 Hz for the 600 MHz and 700 MHz NMR spectrometers, respectively, for 1-4 seconds utilizing a train of 50 ms Gaussian pulses. For off-

resonance spectra, the sample was irradiated at -2000 Hz. The total relaxation delay was 7 seconds. Data acquisition consisted of 128 scans and 32,768 points. The STD-effect was calculated by measuring the intensity of the virstatin aromatic proton peaks in the on- and off-resonance spectra:

$$I_{STD} = \frac{I_0 - I_{SAT}}{I_0} x\ 100 \quad (1)$$

[0098] The STD amplification factor (A_{STD}) values for different protons were fitted by using:

$$A_{STD}(t_{SAT}) = A_{STD-MAX}[1 - e^{(-k_{SAT}t_{SAT})}] \quad (2)$$

where $A_{STD} = I_{STD}\ x\ ligand\ excess.$

[0099] The initial slopes STD-AF_0 (the initial growth rates of the STD amplification factors, which corresponds to A_{STD} at zero t_{SAT}) are obtained from:

$$STD - AF_0 = A_{STD-MAX}\ k_{SAT} \quad (3)$$

where $A_{STD-MAX}$ is the maximal achievable A_{STD} (for a very long saturation time).

[00100] The K_D of virstatin was calculated using Michaelis-Menten kinetics. The STD-AF_0 was plotted as a function of the ligand concentration to construct the binding (Langmuir) isotherm:

$$y = \frac{B_{max}[L]}{[L]+K_D} \quad representing \quad STD - AF_0 = \frac{\alpha_{STD}[L]}{[L]+K_D} \quad (4)$$

Competition studies between virstatin and compounds 5a and 3b

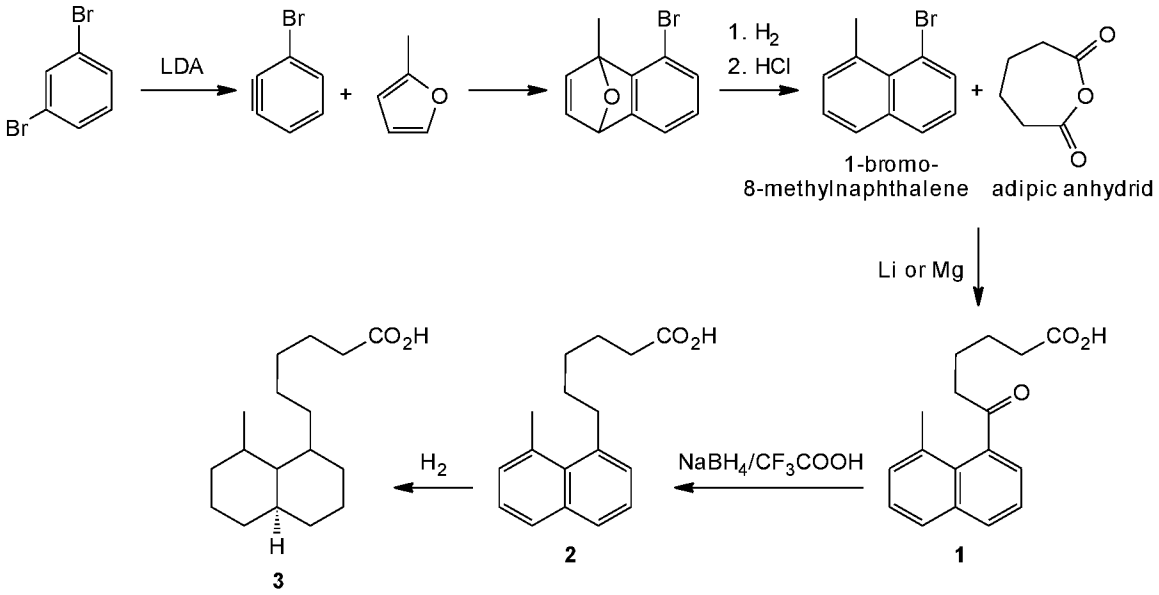
[00101] Virstatin was used as the STD indicator at a concentration of 100 μM. Compounds 5a and 3b were added at a concentration of 20-200 μM. STD spectra were obtained as above, with a saturation time of 3 seconds. The calculation of the competitive inhibition was as follows:

$$K_I = \frac{C_I(K_D - iK_D)}{i(C_L + K_D)} \quad (5)$$

where C_I is the concentration of inhibitor with unknown K_D (compound 5a and 3b), C_L is the concentration of ligand with known K_D (virstatin), K_D is that of virstatin, and i is the inhibition expressed as a fraction: $i = \frac{I_{SAT\ ligand+inhibitor}}{I_{SAT\ ligand\ alone}}$.

Example 1: Design and Synthesis of Compounds

[00102] A synthetic method for preparing exemplary compounds is presented in Scheme 1.



Scheme 1

[00103] Compound **1** was tested in a reporter assay to determine its effect on inhibiting the activity of ToxT. This analysis indicated that compound **1** effectively inhibited the activity of ToxT as determined in a *tcp* reporter assay (Figure 2).

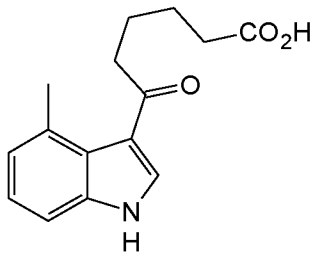
[00104] In light of the activity of compound **1**, this disclosure also includes derivatives of compound **1.** To modify activity, specificity, and/or bioavailability, additional derivatives of compounds **1-3** can be prepared. For example, by varying the anhydride (or a functionalized dicarboxylic acid), the carbon chain linking the naphthalene ring to the carboxylate head group can be lengthened or shortened. Thus, reaction of 1-bromo-8-methylnaphthalene with glutaric anhydride will afford a compound similar to compounds **1-3** having three carbons between the ketone carbonyl and the carboxylate group. Likewise, carrying out the acylation reaction with pimelic acid anhydride will give a compound similar to compounds **1-3** having five carbons between the ketone carbonyl and the carboxylate group. Accordingly, the acylation reaction can be carried out with any suitable dicarboxylic acid, *e.g.*, malonic, succinic, glutaric, adipic, pimelic or suberic acid/anhydride, to achieve a compound having between 1 and 6 carbons between the ketone carbonyl and the carboxylate group.

[00105] Further, the methyl group attached to the naphthalene ring can be substituted with any other alkyl or polar group, either by *de novo* synthesis from a suitable 2-alkylfuran and 3-bromobenzyne (see synthesis of 1-bromo-8-methylnaphthalene in Scheme 1) or by modification of the methyl group in the final product. For example, appropriate oxidation will convert this methyl group to a carboxylate or to a hydroxyl group. Of note, the synthesis of 1-bromo-8-methylnaphthalene automatically gives the isomeric 1-bromo-5-methylnaphthalene, which provides a set of control compounds isomeric to compounds **1-3.**

[00106] The aromatic carbonyl ketone group can be readily reduced with sodium borohydride in trifluoroacetic acid (Gribble, et al. (1978) *Synthesis* 763) or other two-step reduction procedures known in the art. The resulting compounds can be further reduced by catalytic hydrogenation to a derivative of compound **3**. By adjusting the reduction conditions (*e.g.*, Birch reduction), compounds having one reduced ring (*i.e.*, a tetralin analogue [1,2,3,4-tetrahydronaphthalene]) can be prepared.

[00107] As a further derivative, the ketone carbonyl in Compound **1** can be converted to an alkene, so as to "stiffen" the carbon chain.

[00108] In certain embodiments, the naphthalene ring is replace with 4-methylindole, which could be deemed an isostere to 8-methylnaphthalene. Introduction of carboxylate chains onto the indole C-3 position is facile as this position is extremely susceptible to electrophilic substitution. Moreover, the indole double bond is readily reduced, providing additional flexibility for polarity of the basic two-ring structure. This is shown in Compound 4.



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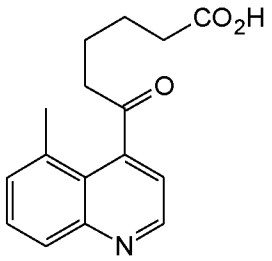
[00109] Similarly, the 8-methylnaphthalene unit can be replaced with the isostere 5-methylquinoline. Once again, the C-4 position of quinoline, like C-3 in indole, is very easily substituted and will provide a ring structure for conversion to derivatives of

compound 1 and, by reduction, to derivatives of compound **3**. This is shown in compound 5, and, for isoquinoline, compound **6**.



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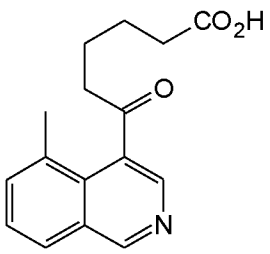

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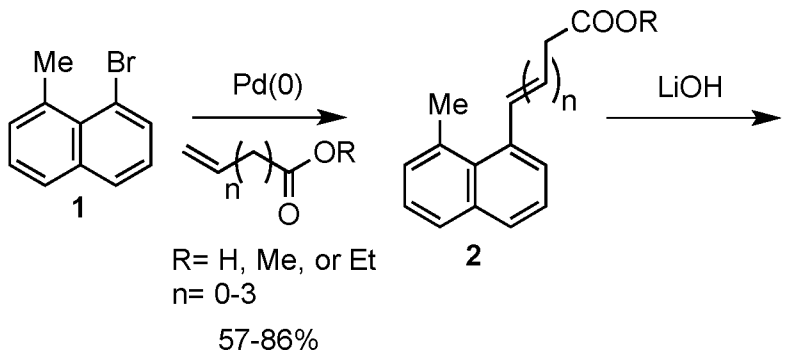
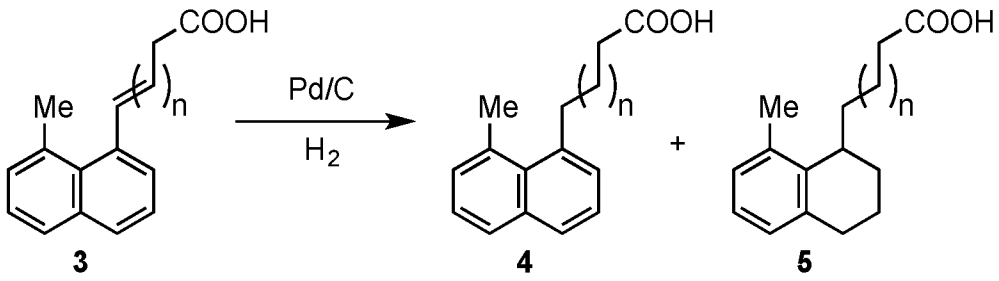
Example 2: Synthesis of Compounds: General Procedure

[00110] As shown below in Scheme 2, the five- and six-step syntheses of the naphthalene 3, 4 and tetralin 5 analogs, respectively, utilized the well-known Mizoroki-Heck reaction (Lemhadri, M., Battace, A., Zair, T., Doucet, H. & Santelli, M. Heck arylations of pent-4-enoates or allylmalonate using a palladium/tetraphosphine catalyst. *Journal of Organometallic Chemistry* 692, 2270-2281 (2007)). The "anchor" for the palladium-catalyzed C-C coupling was 1-bromo-8-methylnaphthalene (1), whose synthesis has been previously optimized (Onyango, E. O., Kelley, A. R., Qian, D. C. & Gribble, G. W. Syntheses of 1-Bromo-8-methylnaphthalene and 1-Bromo-5-methylnaphthalene. *J. Org. Chem.* 80, 5970-5972 (2015)). Following the attachment of the carboxylic acid chain to the naphthalene ring and hydrolysis of the ester 2 (if necessary), controlled catalytic hydrogenation gave the various final products, with the reaction time corresponding to the level of saturation; hydrogenation of the double bond in the carboxylate chain of 3 occurred after ~30 minutes to yield **4**, while prolonged hydrogenation (overnight) yielded **5**.





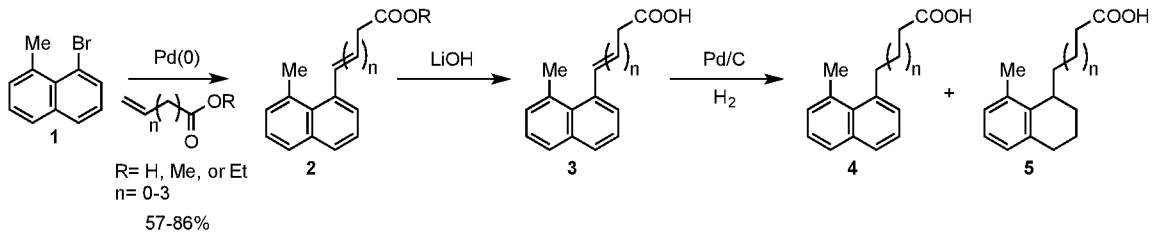
Scheme 2

[00111] Example 3: Synthesis of Compounds: Detailed Procedure

I. General Methods for Synthetic Procedures

[00112] All reactions were conducted in oven-dried glassware under nitrogen gas (N_2). Solvents were reagent grade. All reagents were obtained commercially as reagent grade and, unless otherwise noted, used without further purification. Reactions were monitored by TLC silica gel plates (0.25 mm) and visualized by UV light or *p*-anisaldehyde. Column chromatography was performed using silica gel (60, particle size 40-60 mm). The organic extracts were dried over anhydrous $MgSO_4$. Proton (^1H) and carbon (^{13}C) nuclear magnetic resonance spectra were recorded at 500 or 600 MHz. Chemical shifts are reported in parts per million (ppm, δ) with the residual deuterated solvent as an internal standard (7.26/77.16 ppm for chloroform $^1H/^{13}C$, 5.32/53.84 for methylene chloride $^1H/^{13}C$). Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet. High-resolution mass spectra (HRMS) were obtained employing electron ionization (EI), with TOF as the mass analyzer

II. Synthetic Procedures

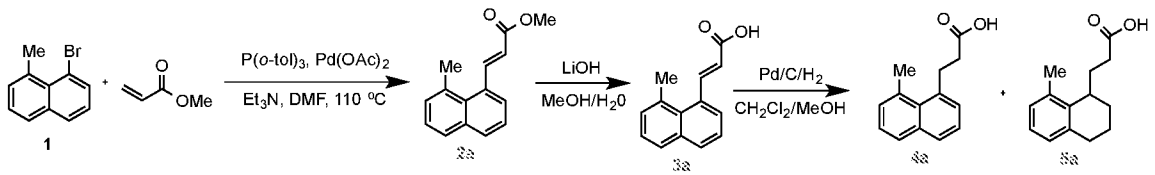


[00113] General procedure 1 (Heck coupling: To a solution of 1-bromo-8-methylnaphthalene (**1**) (1 eq.), palladium(II) acetate (0.05 eq.), tri-*o*-tolylphosphine (0.1 eq.), and triethylamine (10 eq.) in DMF was added alkene (3.5 eq.) dropwise. The

reaction mixture was stirred overnight at 110 °C, then cooled to room temperature and quenched with water. The organic layer was extracted twice with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Purification of the crude mixture via silica gel chromatography and in some cases recrystallization/trituration yielded the corresponding product **2**.

[00114] General procedure 2 (ester hydrolysis): To a solution of **2** (1 eq.) in MeOH/H_20 (3:1) was added LiOH (3 eq.). The mixture was stirred overnight at room temperature. The solution was partially concentrated *in vacuo* and quenched with 1M HCl. The organic layer was extracted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was recrystallized and/or triturated to yield the acid **3**.

[00115] General procedure 3 (hydrogenation: To a solution of **2** or **3** (1 eq.) in CH_2Cl_2/MeOH (1:1) was added 10% Pd-C (0.1 eq.). The mixture was stirred vigorously at room temperature under H_2 for 30 minutes. The mixture was filtered through Celite and concentrated *in vacuo.* Trituration and/or recrystallization resulted in **4** and trace amounts of **5** (or their ester equivalents). For reactions allowed to proceed overnight, only **5** was obtained. In some cases, **5** was conveniently obtained using **4** as SM.

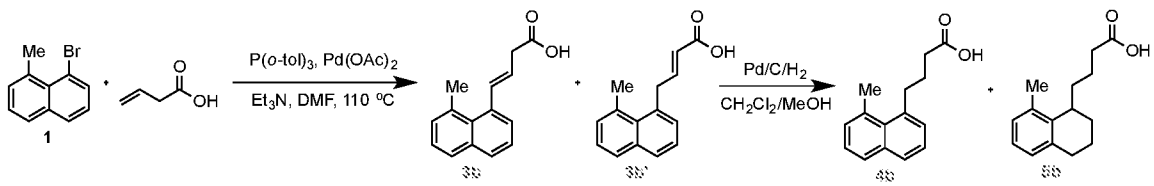


[00116] **(*E*)-3-(8-Methylnaphthalen-1-yl)acrylic acid (3a)**: General procedure 1 was followed using **1** (60 mg, 0.271 mmol), $Pd(OAc)_2$ (6.09 mg, 0.0271 mmol), P(*o*-tol)$_3$ (16.52 mg, 0.0543 mmol), Et_3N (0.38 mL, 2.714 mmol), DMF (0.1M, 3 mL), and methyl acrylate (0.09 mL, 0.950 mmol). The crude mixture, a green-brown oil, was purified via silica gel chromatography (20:1 hexanes/ethyl acetate) to afford 2a (53 mg, 86%) as colorless oil. General procedure 2 was followed using 2a (53 mg, 0.234 mmol), MeOH/H_20 (3:1; 3 mL), and LiOH (29.48 mg, 0.703 mmol) to yield 3a as a white solid (48 mg, 97%), which was further purified by trituration with hexanes. TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.30; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.82 (d, *J* = 15.6 Hz, 1H), 7.87 (dd, *J* = 8.1, 1.1 Hz, 1H), 7.73 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.52 (d, *J* = 7.0 Hz, 1H), 7.45 – 7.36 (m, 3H), 6.24 (d, *J* = 15.5 Hz, 1H), 2.87 (s, 3H); ^{13}C NMR (600 MHz,

$CDCl_3$): δ 172.0, 152.0, 135.0, 134.9, 134.3, 131.6, 131.5, 130.4, 128.1, 127.7, 126.1, 125.1, 118.6, 25.7; HRMS (*m/z*): $[M]^+$ calcd. for $C_{14}H_{12}O_2$, 212.0837; found, 212.0841.

[00117] **3-(8-Methylnaphthalen-1-yl)propanoic acid (4a)**: General procedure 3 was followed using 3a (46.7 mg, 0.220 mmol), CH_2Cl_2/MeOH (1:1; 2.5 mL), and 10% Pd-C (23.03 mg, 0.022 mmol). The crude was triturated with MeOH to yield 4a as a pure white solid in quantitative yield. (The trace amounts of the tetralin derivative dissolved in MeOH, while the naphthalene did not). TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.29; ^1H NMR (500 MHz, $CDCl_3$): δ 7.75 – 7.70 (m, 2H), 7.38 – 7.29 (m, 4H), 3.66 – 3.62 (m, 2H), 2.93 (s, 3H), 2.73 – 2.70 (m, 2H); ^{13}C NMR (600 MHz, $CDCl_3$): δ 177.8, 137.5, 135.9, 134.2, 132.0, 130.5, 129.0, 128.5, 125.3, 125.2, 37.0, 31.9, 25.7; HRMS (*m/z*): $[M]^+$ calcd. for $C_{14}H_{14}O_2$, 214.0994; found, 214.0999.

[00118] **3-(8-Methyl-1,2,3,4-tetrahydronaphthalen-1-yl)propanoic acid (5a)**: General procedure 3 was followed using 4a (40 mg, 0.187 mmol), CH_2Cl_2/MeOH (1:1; 2 mL), and 10% Pd-C (19.54 mg, 0.0187 mmol). The reaction yielded 5a as yellowish oil (39 mg, 96%). TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.34; ^1H NMR (600 MHz, $CDCl_3$): δ 7.04 – 6.91 (m, 3H), 2.82 – 2.74 (m, 3H), 2.55 – 2.41 (m, 2H), 2.31 (s, 3H), 1.93 – 1.67 (m, 6H); ^{13}C NMR (600 MHz, $CDCl_3$): δ 178.1, 139.4, 136.5, 136.1, 128.1, 127.3, 125.8, 34.0, 32.2, 29.4, 28.8, 25.4, 19.1, 17.7; HRMS (*m/z*): $[M]^+$ calcd. for $C_{14}H_{18}O_2$, 218.1307; found, 218.1310.

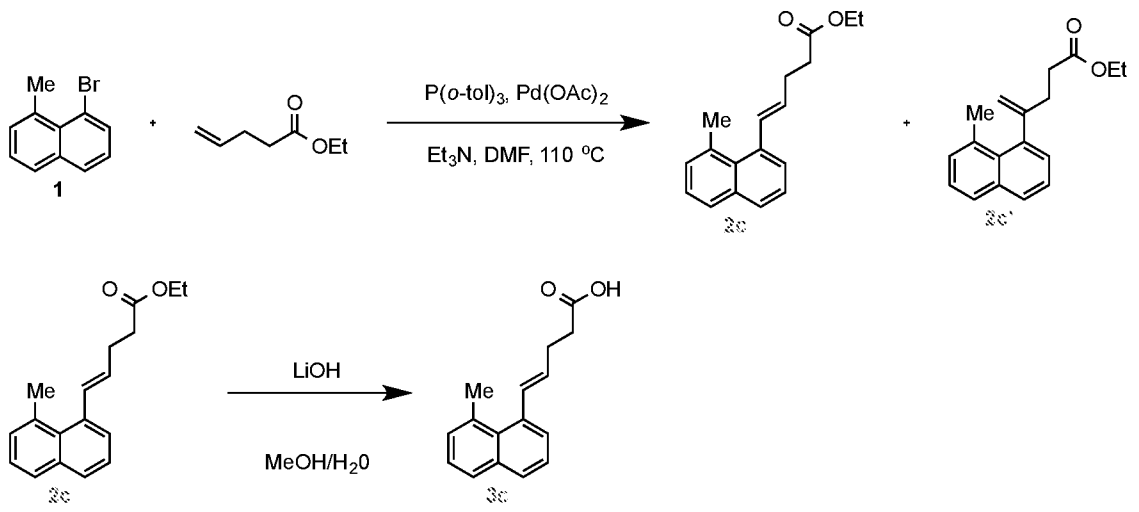


(*E*)-4-(8-Methylnaphthalen-1-yl)but-3-enoic acid (3b): General procedure 1 was followed using **1** (100 mg, 0.452 mmol), $Pd(OAc)_2$ (5.08 mg, 0.0226 mmol), P(*o*-tol)$_3$ (13.77 mg, 0.0452 mmol), Et_3N (0.63 mL, 4.52 mmol), DMF (0.1M, 2.5 mL), and but-3-enoic acid (0.13 mL, 1.583 mmol). The crude mixture, an orange oil, was purified via silica gel chromatography (20:1 to 2:1 hexanes/ethyl acetate) to afford a mixture of 3b and 3b'. Further purification by trituration with hot hexanes left the pure product 3b as a white solid (58.7 mg, 57%). TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.23; ^1H NMR (500 MHz, CD_2Cl_2): δ 7.77 – 7.74 (m, 1H), 7.68 (d, *J* = 7.9 Hz, 1H), 7.44 (d, *J* = 15.6 Hz, 1H),

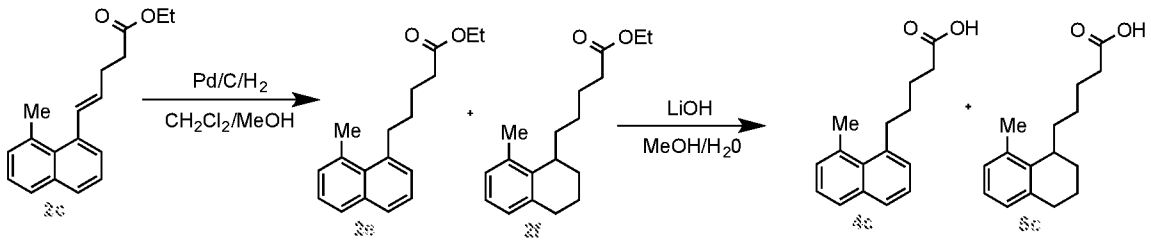
7.39 – 7.27 (m, 4H), 5.97 – 5.90 (m, 1H), 3.37 (dd, J = 7.0, 1.5 Hz, 2H), 2.84 (s, 3H); ^{13}C NMR (600 MHz, CD_2Cl_2): δ 175.7, 138.5, 137.6, 135.7, 135.3, 131.7, 129.8, 129.4, 128.2, 127.7, 125.7, 125.5, 122.6, 38.0, 26.0; HRMS (*m/z*): $[M]^+$ calcd. for $C_{15}H_{14}O_2$, 226.0994; found, 226.0995. The structure of 3b was unambiguously assigned based on HMBC NMR analysis.

[00119] **4-(8-Methylnaphthalen-1-yl)butanoic acid (4b)** : General procedure 3 was followed using a mixture of 3b and 3b' (52 mg, 0.230 mmol), CH_2Cl_2/MeOH (1:1; 2.5 mL), and 10% Pd-C (24.05 mg, 0.023 mmol). The crude orange solid was triturated with MeOH to yield 4b as a pure white solid (50 mg, 96%)._TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.28; ^1H NMR (500 MHz, $CDCl_3$): δ 7.72 – 7.69 (m, 2H), 7.36 – 7.26 (m, 4H), 3.31 (t, J = 7.8 Hz, 2H), 2.91 (s, 3H), 2.45 (t, J = 7.3 Hz, 2H), 2.03 – 1.97 (m, 2H); ^{13}C NMR (600 MHz, $CDCl_3$): δ 178.2, 138.7, 136.0, 134.4, 132.1, 130.3, 129.4, 128.6, 128.4, 125.1, 36.2, 33.4, 28.0, 25.5; HRMS (*m/z*): $[M]^+$ calcd. for $C_{15}H_{16}O_2$, 228.1150; found, 228.1149.

[00120] **4-(8-Methyl-1,2,3,4-tetrahydronaphthalen-1-yl)butanoic acid (5b)**: General procedure 3 was followed using 4b (45 mg, 0.197 mmol), CH_2Cl_2/MeOH (1:1; 2 mL), and 10% Pd-C (20.63 mg, 0.0197 mmol). The reaction yielded 5b as colorless oil in quantitative yield. TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.35; ^1H NMR (600 MHz, $CDCl_3$): δ 7.02 – 6.90 (m, 3H), 2.82 – 2.73 (m, 3H), 2.46 – 2.36 (m, 2H), 2.28 (s, 3H), 1.92 – 1.43 (m, 8H); ^{13}C NMR (600 MHz, $CDCl_3$): δ 178.3, 140.2, 136.5, 136.0, 128.1, 127.2, 125.6, 34.6, 33.9, 33.7, 29.6, 25.6, 23.3, 19.1, 17.8; HRMS (*m/z*): $[M]^+$ calcd. for $C_{15}H_{20}O_2$, 232.1463; found, 232.1470.

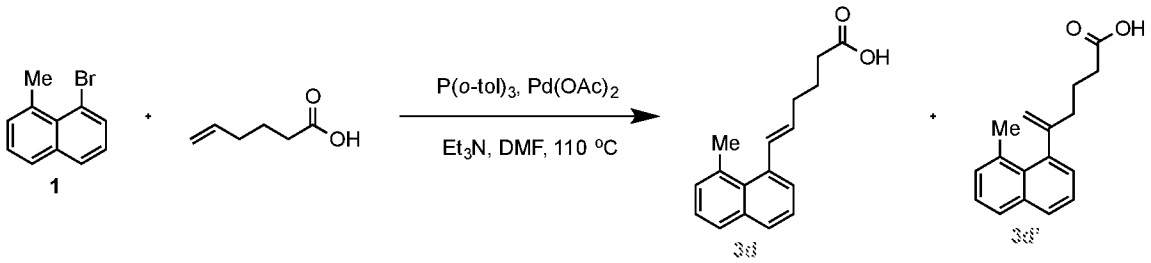


[00121] **(*E*)-5-(8-Methylnaphthalen-1-yl)pent-4-enoic acid (3c)**: General procedure 1 was followed using **1** (1.3 g, 5.88 mmol), $Pd(OAc)_2$ (66.01 mg, 0.294 mmol), P(*o*-tol)$_3$ (179.0 mg, 0.588 mmol), Et_3N (8.17 mL, 58.80 mmol), DMF (0.1M, 60 mL), and ethyl pent-4-enoate (2.93 mL, 20.58 mmol). The crude mixture, a dark brown oil, was purified via silica gel chromatography (50:1 hexanes/ethyl acetate) to afford 2c (1.05 g, 67%) as orange oil. General procedure 2 was followed using 2c (100 mg, 0.373 mmol), MeOH/H_20 (3:1; 3 mL), and LiOH (46.91 mg, 1.118 mmol). The crude was triturated with hexanes to yield 3c as a white solid in quantitative yield. TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.33; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 7.74 (dd, *J* = 7.6, 1.9 Hz, 1H), 7.68 (d, *J* = 7.9 Hz, 1H), 7.37 – 7.29 (m, 4H), 7.25 (d, *J* = 7.0 Hz, 1H), 5.88 – 5.82 (m, 1H), 2.84 (s, 3H), 2.65 – 2.62 (m, 4H); ^{13}C NMR (600 MHz, $CDCl_3$): δ 179.4, 137.8, 135.8, 135.4, 135.0, 131.5, 129.5, 129.0, 128.9, 127.9, 127.5, 125.4, 125.2, 33.7, 28.2, 26.0; HRMS (*m/z*): $[M]^+$ calcd. for $C_{16}H_{16}O_2$, 240.1150; found, 240.1151. **2c' is identified based on the signature doublet with a large coupling constant around 5.20 ppm.*



[00122] **5-(8-Methylnaphthalen-1-yl)pentanoic acid (4c)**: General procedure 3 was followed using 2c (106 mg, 0.395 mmol), CH_2Cl_2/MeOH (1:1; 4 mL), and 10% Pd-C (41.34 mg, 0.0395 mmol) to yield 2e as pale yellow oil (100 mg, 94%), which was used in the next step without further purification. General procedure 2 was followed using 2e (100 mg, 0.370 mmol), MeOH/H_20 (3:1; 3 mL), and LiOH (46.56 mg, 1.110 mmol) to yield a pale yellow solid (83 mg, 93%). The crude product was recrystallized in MeOH to yield 4c as a white solid. TLC (hexanes:EtOAc, 3:1 v/v): R_f = 0.30; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.70 (d, *J* = 7.7 Hz, 2H), 7.35 – 7.26 (m, 4H), 3.27 (t, *J* = 7.7 Hz, 2H), 2.90 (s, 3H), 2.40 (t, *J* = 7.3 Hz, 2H), 1.82 – 1.76 (m, 2H), 1.73 – 1.69 (m, 2H); ^{13}C NMR (600 MHz, CD_2Cl_2): δ 178.4, 140.1, 136.2, 135.0, 132.4, 130.3, 129.3, 128.5, 128.4, 125.3, 37.0, 33.9, 33.2, 25.6, 24.9; HRMS (*m/z*): $[M]^+$ calcd. for $C_{16}H_{18}O_2$, 242.1307; found, 242.1309.

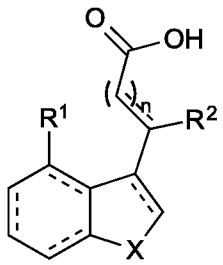
[00123] 5-(8-Methyl-1,2,3,4-tetrahydronaphthalen-1-yl)pentanoic acid (5c): General procedure 3 was followed using 2c (88 mg, 0.328 mmol), CH_2Cl_2/MeOH (1:1; 3 mL), and 10% Pd-C (34.33 mg, 0.0328 mmol) to yield 2f as pale yellow oil in quantitative yield, which was used in the next step without further purification. General procedure 2 was followed using 2f (90 mg, 0.328 mmol), MeOH/H_20 (3:1; 3 mL), and LiOH (41.32 mg, 0.985 mmol) to yield 5c as pale yellow oil (71 mg, 88%). TLC (hexanes:EtOAc, 3:1 v/v) R_f= 0.43; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 6.98 – 6.86 (m, 3H), 2.79 – 2.70 (m, 3H), 2.41 – 2.35 (m, 2H), 2.27 (s, 3H), 1.87 – 1.39 (m, 10H); ^{13}C NMR (600 MHz, CD_2Cl_2): δ 178.0, 140.9, 136.7, 136.2, 128.1, 127.3, 125.6, 34.9, 34.2, 34.0, 29.8, 27.8, 25.9, 25.2, 19.1, 18.1; HRMS (*m/z*): $[M]^+$ calcd. for $C_{16}H_{22}O_2$, 246.1620; found, 246.1619.



[00124] (*E*)-6-(8-Methylnaphthalen-1-yl)hex-5-enoic acid (3d): General procedure 1 was followed using **1** (100 mg, 0.452 mmol), $Pd(OAc)_2$ (5.08 mg, 0.0226 mmol), P(*o*-tol)$_3$ (13.77 mg, 0.0452 mmol), Et_3N (0.63 mL, 4.52 mmol), DMF (0.1M, 2.5 mL), and hex-5-enoic acid (0.19 mL, 1.583 mmol). The crude mixture, an orange oil, was purified via silica gel chromatography (20:1 to 2:1 hexanes/ethyl acetate) to afford a mixture of hex-5-enoic acid, 3d, and 3d' *. Further purification by trituration first with hot hexanes (to remove the hexenoic acid) and second by EtOAc (to remove 3d') left the pure product 3d as a white solid (65.5 mg, 57%). TLC (hexanes:EtOAc, 3:1 v/v): R_f= 0.32; ^{1}H NMR (500 MHz, CD_2Cl_2): δ 7.73 (dd, *J* = 7.3, 2.1 Hz, 1H), 7.67 (d, *J* = 7.9 Hz, 1H), 7.37 – 7.26 (m, 5H), 5.87 – 5.80 (m, 1H), 2.86 (s, 3H), 2.47 (t, *J* = 7.5 Hz, 2H), 2.38 – 2.33 (m, 2H), 1.92 – 1.86 (m, 2H); ^{13}C NMR (600 MHz, CD_2Cl_2): δ 177.8, 138.5, 135.9, 135.5, 135.3, 131.8, 130.9, 129.6, 129.0, 128.0, 127.7, 125.6, 125.5, 33.5, 32.7, 26.2, 24.7; HRMS (*m/z*): $[M]^+$ calcd. for $C_{17}H_{18}O_2$, 254.1307; found, 254.1307. *3d' is identified based on the signature doublet with a large coupling constant around 5.2 ppm.

CLAIMS

1. A compound having the structure of Formula I, or a pharmaceutically acceptable salt thereof:



Formula I

wherein

R^1 is hydrogen (H) or C_1-C_6 alkyl, C_1-C_6 alkenyl, C_4-C_6 aryl, halo, -CF_3, -OCH_3, -NO_2, -CN, -OH, -NMe_2, -COOH, or -$COOCH_3$;

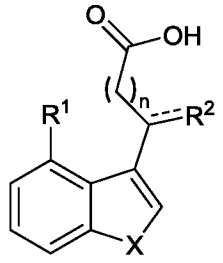
R^2 is hydrogen (H), alkylidenyl (=CH_2), or oxo (=O);

X is –CH_2CH_2-, –CH=CH-, -NH-, -C=NH-, -N=C-, -CH_2-NH- or -NH-CH_2-;

n is 0-6; and

dashed lines represent bonds that are independently present or absent.

2. The compound of claim 1 having the structure of Formula Ia, or a pharmaceutically acceptable salt thereof:



Ia

wherein

R^1 is selected from the group consisting of hydrogen, -OH and C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group;

R^2 is hydrogen, attached by a single bond, or R^2 is $=CH_2$ or =O, attached by a double bond;

X is selected from the group consisting of –CH=CH-, -NH-, -CH=N-, and -N=CH-;

n is selected from 1, 2, 3 and 4; and

a dashed line represents a bond that is either present or absent.

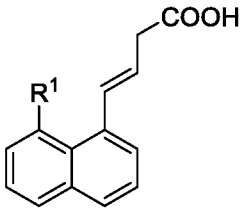
3. The compound of claim 2, wherein R^1 is C_1-C_6 alkyl.

4. The compound of claim 3, wherein R^1 is selected from the group consisting of methyl, ethyl, propyl, butyl, pentyl, and hexyl.

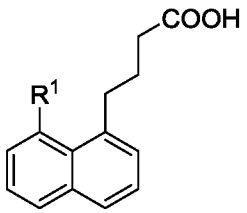
5. The compound of claim 2, wherein R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is substituted with an –OH group.

6. The compound of claim 5, wherein R^1 is selected from the group consisting of $–CH_2OH$, $–CH_2CH_2OH$, $CH_2CH_2CH_2OH$, and $–CH_2CH_2$ CH_2CH_2OH.

7. The compound of claim 2 having a structure selected from the following:



and



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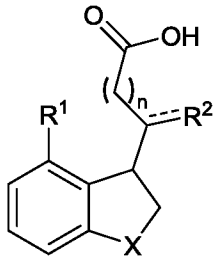
Ia01 Ia02

wherein R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optinally substituted with an –OH group.

8. The compound of claim 1 having the structure of Formula Ib, or a pharmaceutically acceptable salt thereof:



Ib

wherein

R^1 is selected from the group consisting of hydrogen, hydroxyl and C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group;

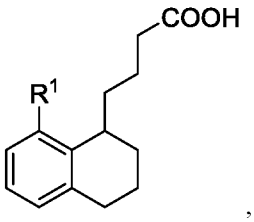
R^2 is hydrogen, attached by a single bond, or R^2 is $=CH_2$ or =O, attached by a double bond;

X is selected from the group consisting of -NH-, $-CH_2-CH_2-$, $-CH_2-NH-$ and $-NH-CH_2-$;

n is selected from 1, 2, 3 and 4; and

a dashed line represents a bond that is either present or absent.

9. The compound of claim 8 having a structure selected from the following:

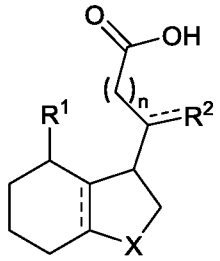


,

Ib01

wherein R^1 is C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is substituted with an –OH group.

10. The compound of claim 1 having the structure of Formula Ic, or a pharmaceutically acceptable salt thereof:



Ic

wherein

R^1 is selected from the group consisting of hydrogen, hydroxyl and C_1-C_6 alkyl, wherein the C_1-C_6 alkyl is optionally substituted with an –OH group;

R^2 is hydrogen, attached by a single bond, or R^2 is $=CH_2$ or =O, attached by a double bond;

X is $-CH_2-CH_2-$; n is selected from 1, 2, 3 and 4; and

each dashed line independently represents a bond that is either present or absent.

11. The compound of any one of the preceding claims having a structure selected from Table 1, or a pharmaceutically acceptable salt thereof.

12. The compound of claim 6, selected from the group consisting of compounds 1, 2, 3, 4, 3a, 3b, 3c, 3d, 4a, 4b, 4c, 5a, 5b and 5c.

13. The compound of any one of the preceding claims, wherein the compound binds to the regulatory pocket of ToxT with a K_d of less than about 300 μM.

14. A compound comprising a carboxylate moiety capable of interacting with Lys31 of the N-terminal domain and Lys230 of the C-terminal domain of the fatty acid binding region of ToxT, and a fused bicyclic moiety.

15. The compound of claim 14, wherein the fused bicyclic moiety is selected from the

group consisting of naphthalene, 1,2,3,4-tetrahydronaphthalene, 1,2,3,4,5,6,7,8-octahydronaphthalene, decalin, isoquinoline, quinoline,

16. The compound of any one of claims 14-15, wherein the fused bicyclic moiety is capable of interacting with Phe22 of the fatty acid binding region of ToxT.

17. The compound of any one of claims 14-16, wherein the compound binds to the regulatory pocket of ToxT with a K_d of less than about 300 μM.

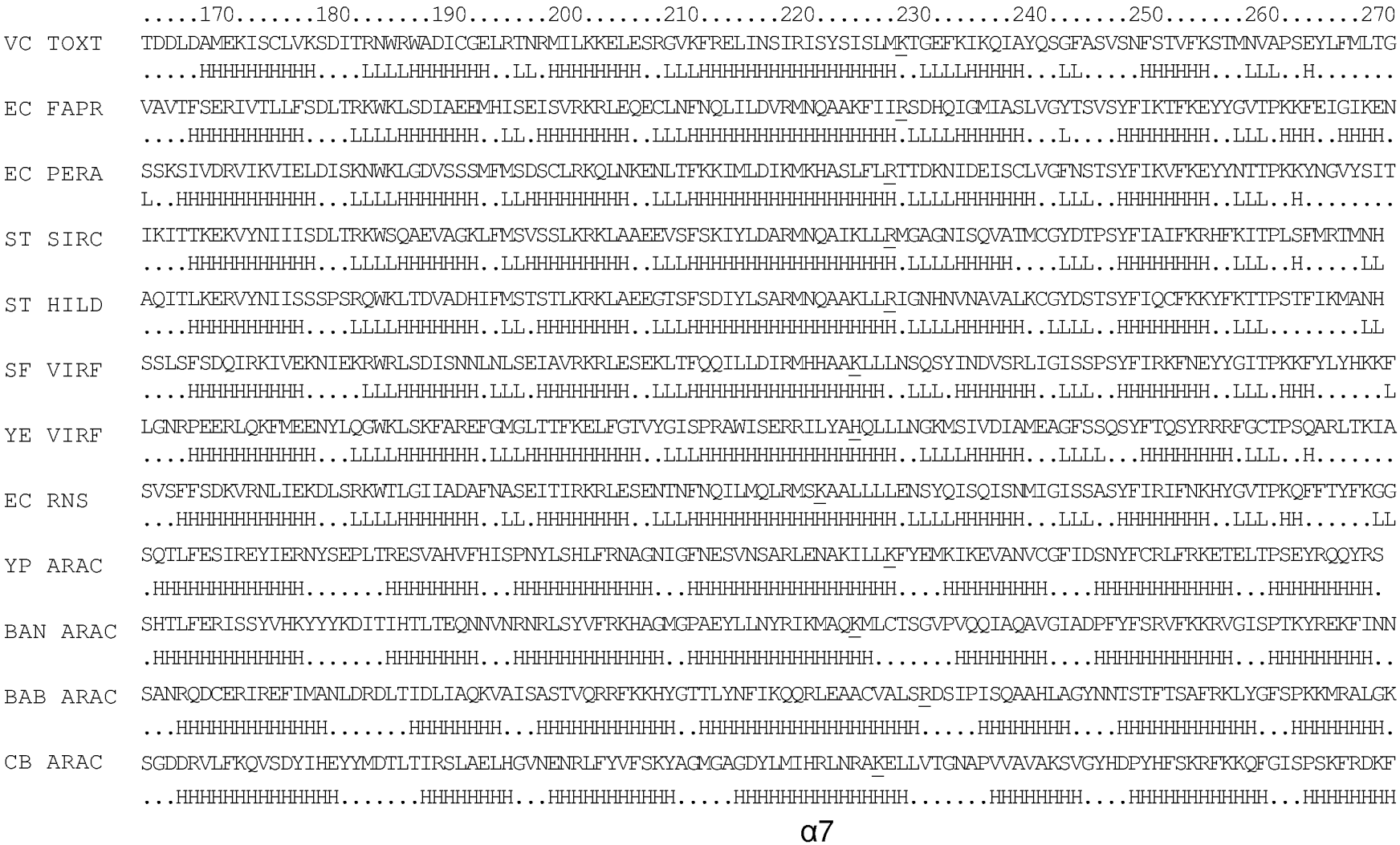
18. A pharmaceutical composition comprising a compound of any one of claims 1-17, and a pharmaceutically acceptable carrier.

19. A method for decreasing expression of a bacterial virulence factor comprising contacting a bacterium that expresses an A/X regulatory protein with a compound according to any one of claims 1-17, such that the expression of a virulence factor by said bacterium is decreased.

20. The method of claim 19, wherein the bacterium is *Vibrio cholerae, Escherichia coli*, *Shigella flexneri*, *Yersinia enterocolitica*, *Yersinia pestis, Brucella abortus, Salmonella typhi*, *Bacillus anthracis, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus* or *Salmonella typhimurium.*

21. A method for preventing, mitigating, or treating an infection by a bacterium that expresses an A/X regulatory protein comprising administering to a subject in need thereof an effective amount of a compound according to any one of claims 1-17, or a pharmaceutical composition thereof, such that the infection is prevented, mitigated, or treated.

22. The method of claim 21, wherein the bacterium is *Vibrio cholerae, Escherichia coli*, *Shigella flexneri*, *Yersinia enterocolitica*, *Yersinia pestis, Brucella abortus, Salmonella typhi*, *Bacillus anthracis, Clostridium botulinum, Listeria monocytogenes, Staphylococcus aureus* or *Salmonella typhimurium.*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EC FAPR
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....HHHHHHHHHH....LLLLHHHHHHH..LL.HHHHHHHH..LLLHHHHHHHHHHHHHHHHH.LLLLHHHHHH...L....HHHHHHHH..LLL.HHH..HHHH.
EC PERA
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L..HHHHHHHHHHH...LLLLHHHHHHH..LLHHHHHHHHH..LLLHHHHHHHHHHHHHHHHH.LLLLHHHHHH..LLL..HHHHHHHH..LLL..H........
ST SIRC
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....HHHHHHHHHH...LLLLHHHHHH..LLHHHHHHHH..LLLHHHHHHHHHHHHHHHHH.LLLLHHHH....LLL..HHHHHHH..LLL..H.....LL
ST HILD
AQITLKERVYNIISSSPSRQWKLTDVADHIFMSTSTLKRKLAEEGISFSDIYLSARMNQAAKLLRIGNHNVNAVALKCGYDSTSYFIQCFKKYFKTTPSTFIKMANH
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SF VIRF
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YE VIRF
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YP ARAC
SQTLFESIREYIERNYSEPLTRESVAHVFHISPNYLSHLFRNAGNIGFNESVNSARLENAKILLKFYEMKIKEVANVCGFIDSNYFCRLFRKETELTPSEYRQQYRS
.HHHHHHHHHHHH.......HHHHHHH...HHHHHHHHHHH...HHHHHHHHHHHHHHHHH....HHHHHHHH....HHHHHHHHHHH...HHHHHHHH.
BAN ARAC
SHTLFERISSYVHKYYYKDITIHTLTEQNNVNRNRLSYVFRKHAGMGPAEYLLNYRIKMAQKMLCTSGVPVQQIAQAVGIADPFYFSRVFKKRVGISPTKYREKFINN
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BAB ARAC
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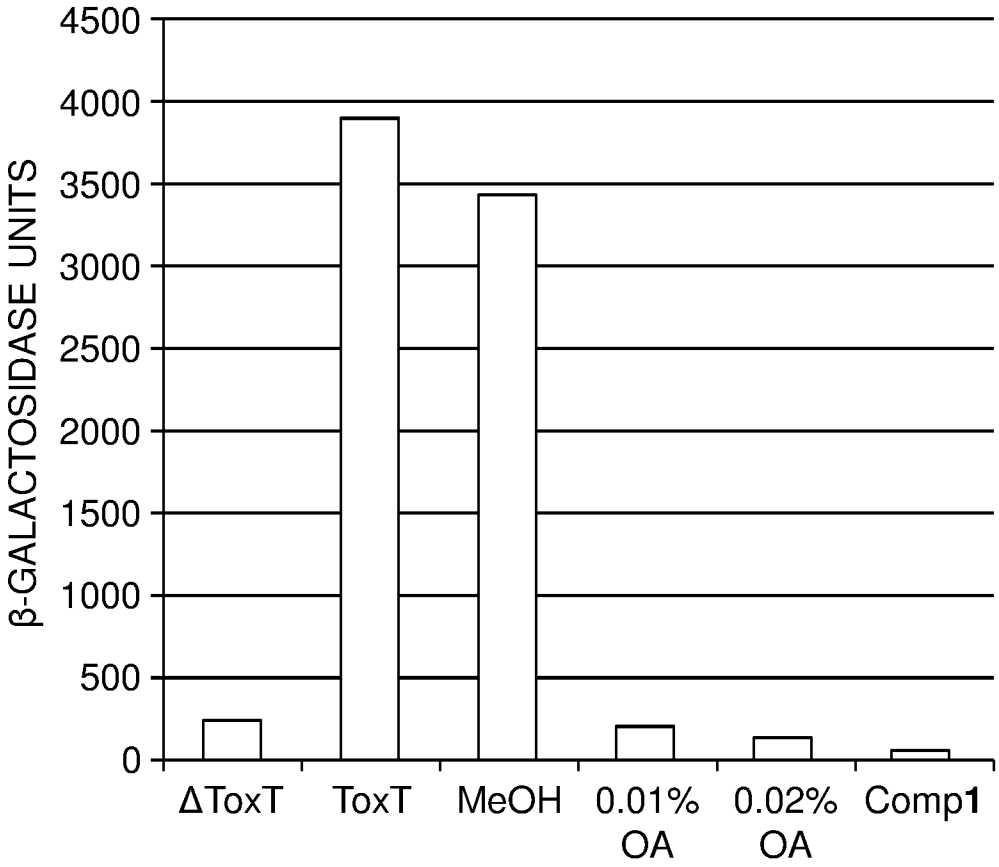
α7

FIG. 1


4500
4000
3500
3000
2500
2000
1500
1000
500
0
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ΔToxT
ToxT
MeOH
0.01% OA
0.02% OA
Comp1

FIG. 2

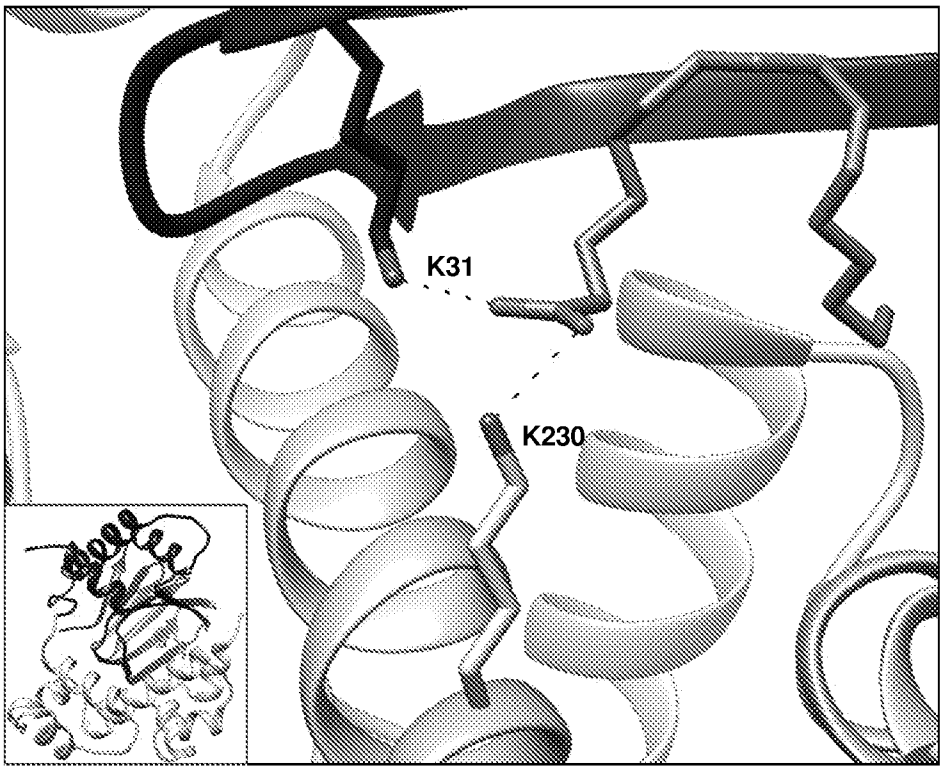

FIG. 3

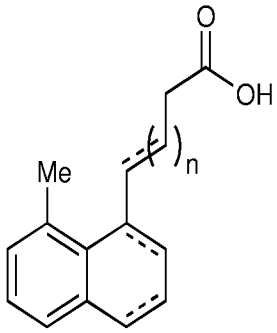

FIG. 4A

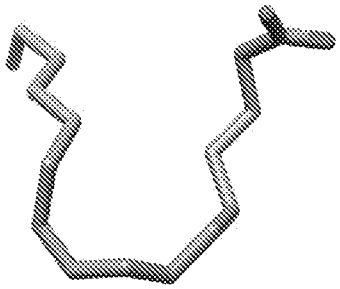

FIG. 4B

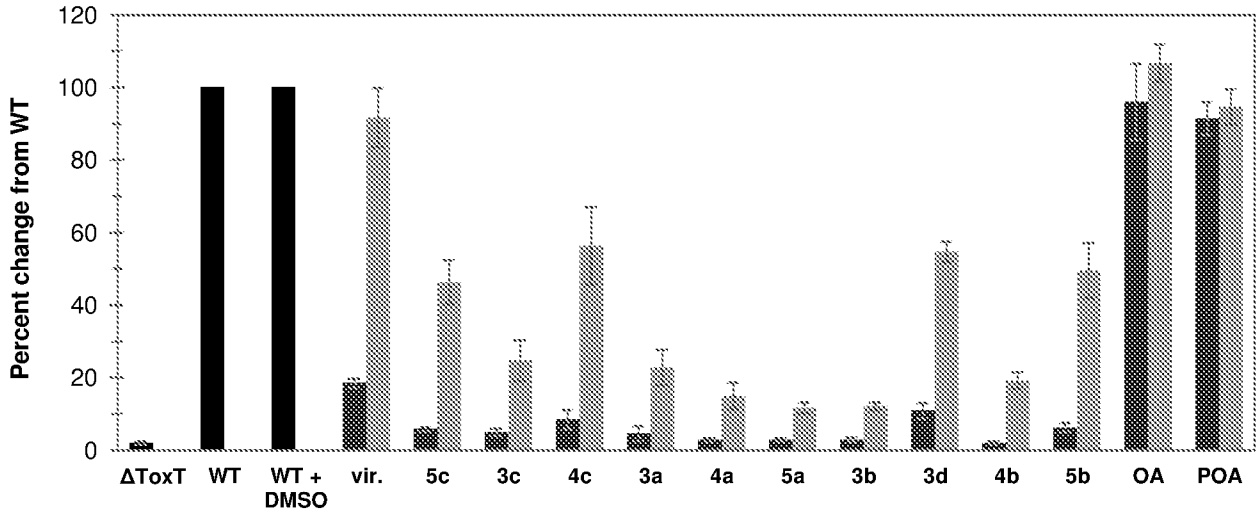

FIG. 5A

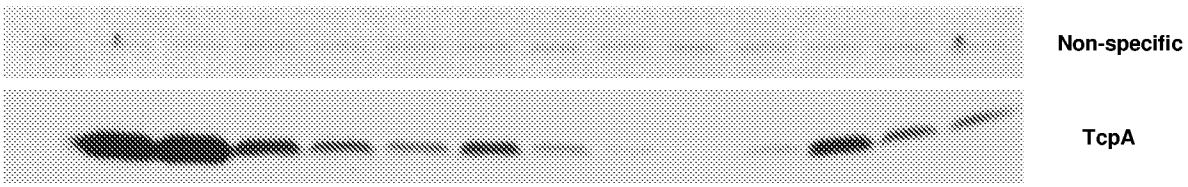

FIG. 5B

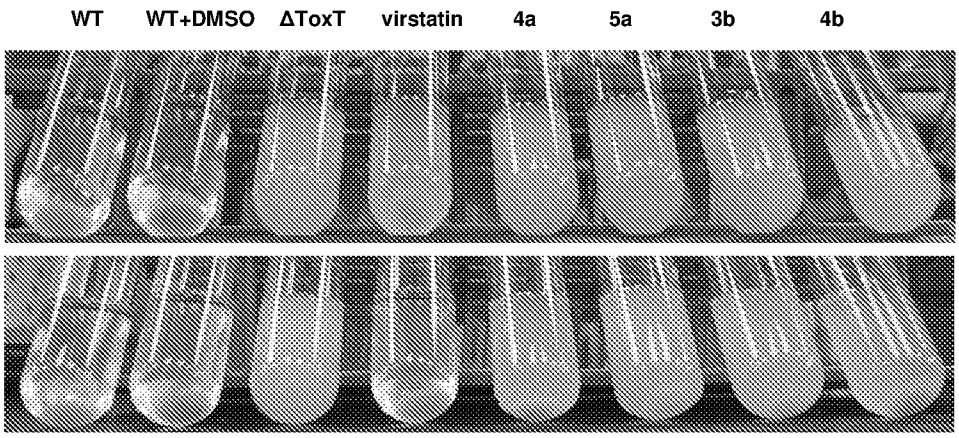

FIG. 5C

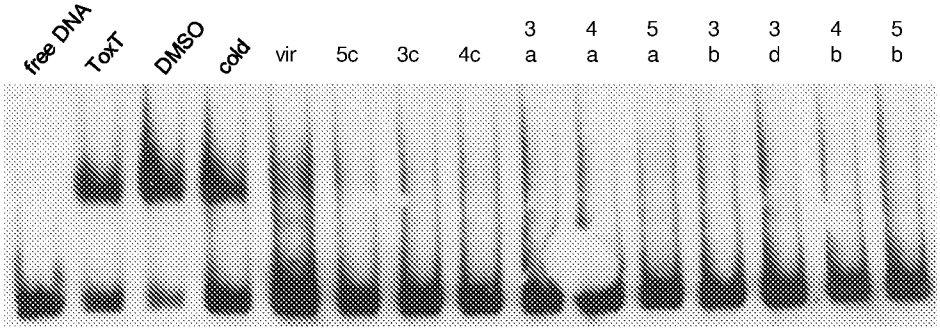

FIG. 6A

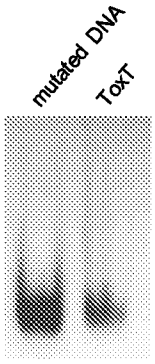

FIG. 6B

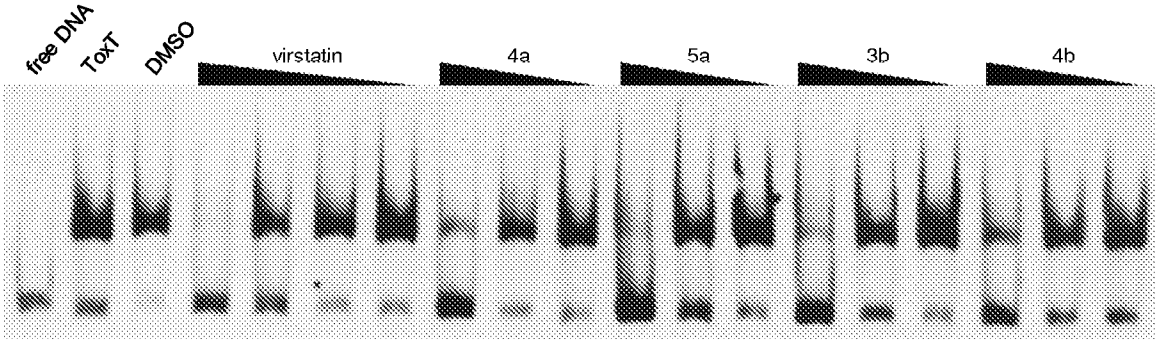

FIG. 6C

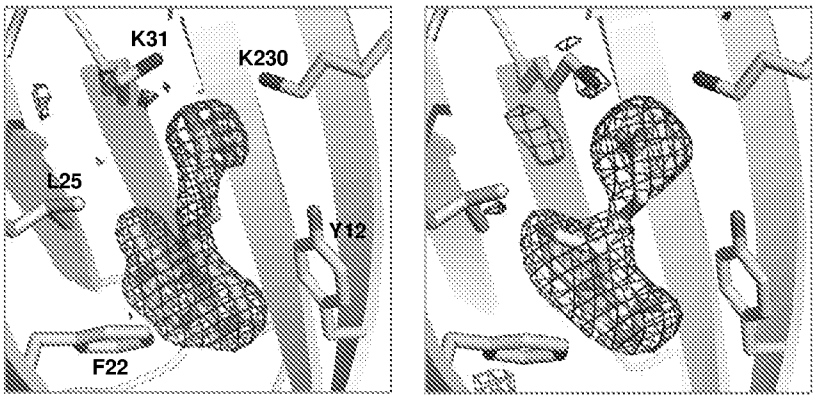

FIG. 7A

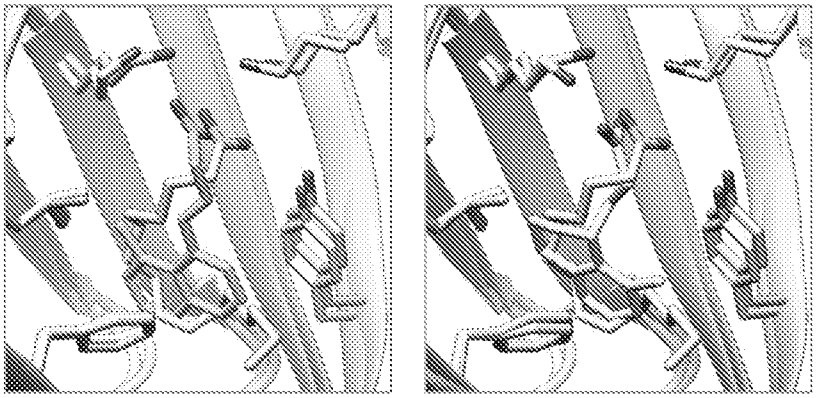

FIG. 7B

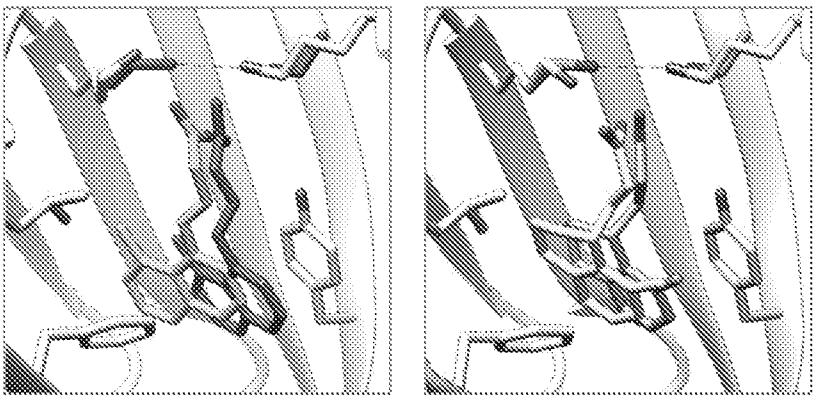

FIG. 7C

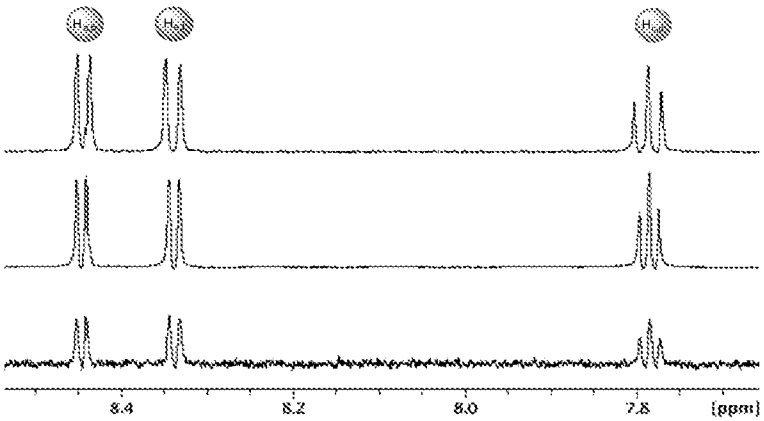

FIG. 8A

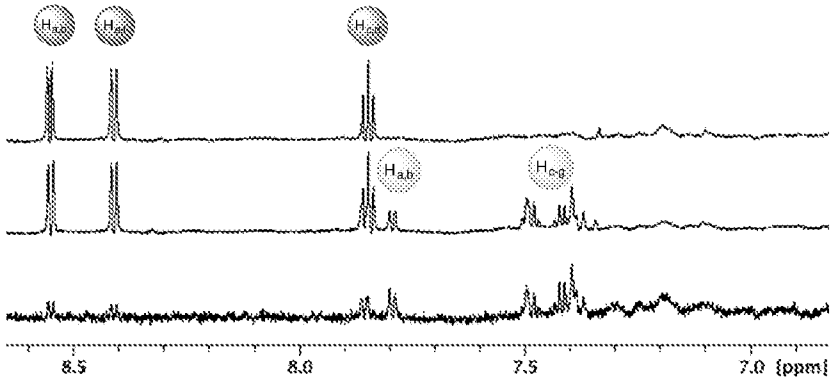

FIG. 8B

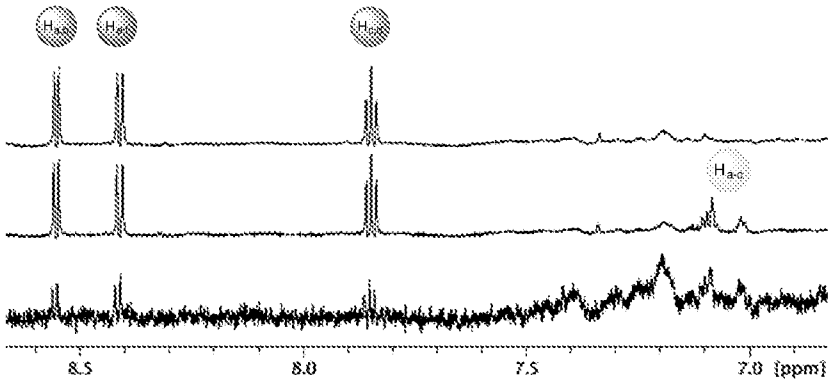

FIG. 8C

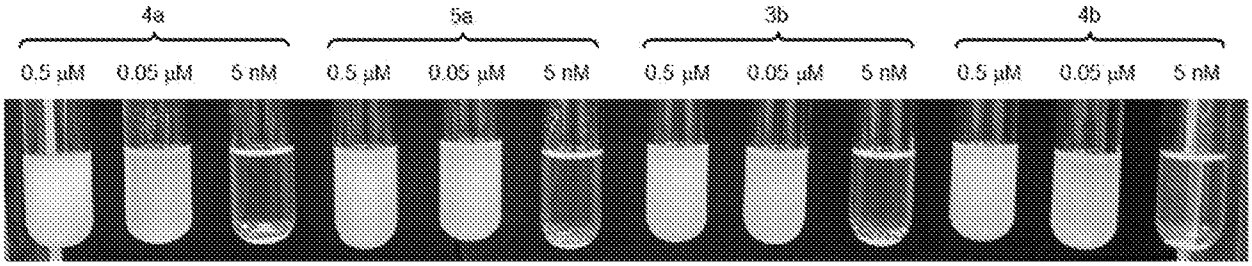
4a
5a
3b
4b
0.5 μM
0.05 μM
5 nM
0.5 μM
0.05 μM
5 nM
0.5 μM
0.05 μM
5 nM
0.5 μM
0.05 μM
5 nM

FIG. 9A

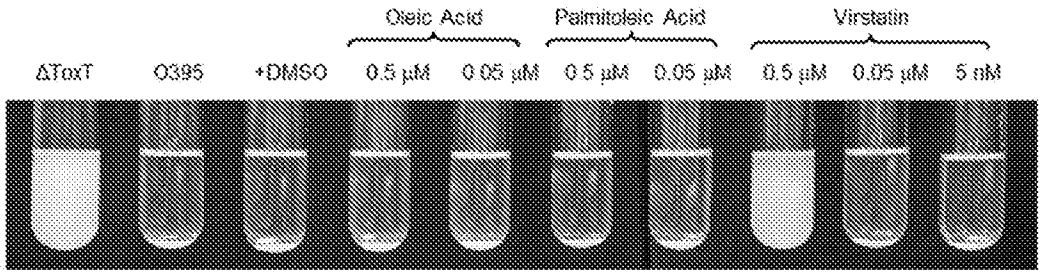
Oleic Acid
Palmitoleic Acid
Virstatin
ΔToxT
O395
+DMSO
0.5 μM
0.05 μM
0.5 μM
0.05 μM
0.5 μM
0.05 μM
5 nM

FIG. 9B

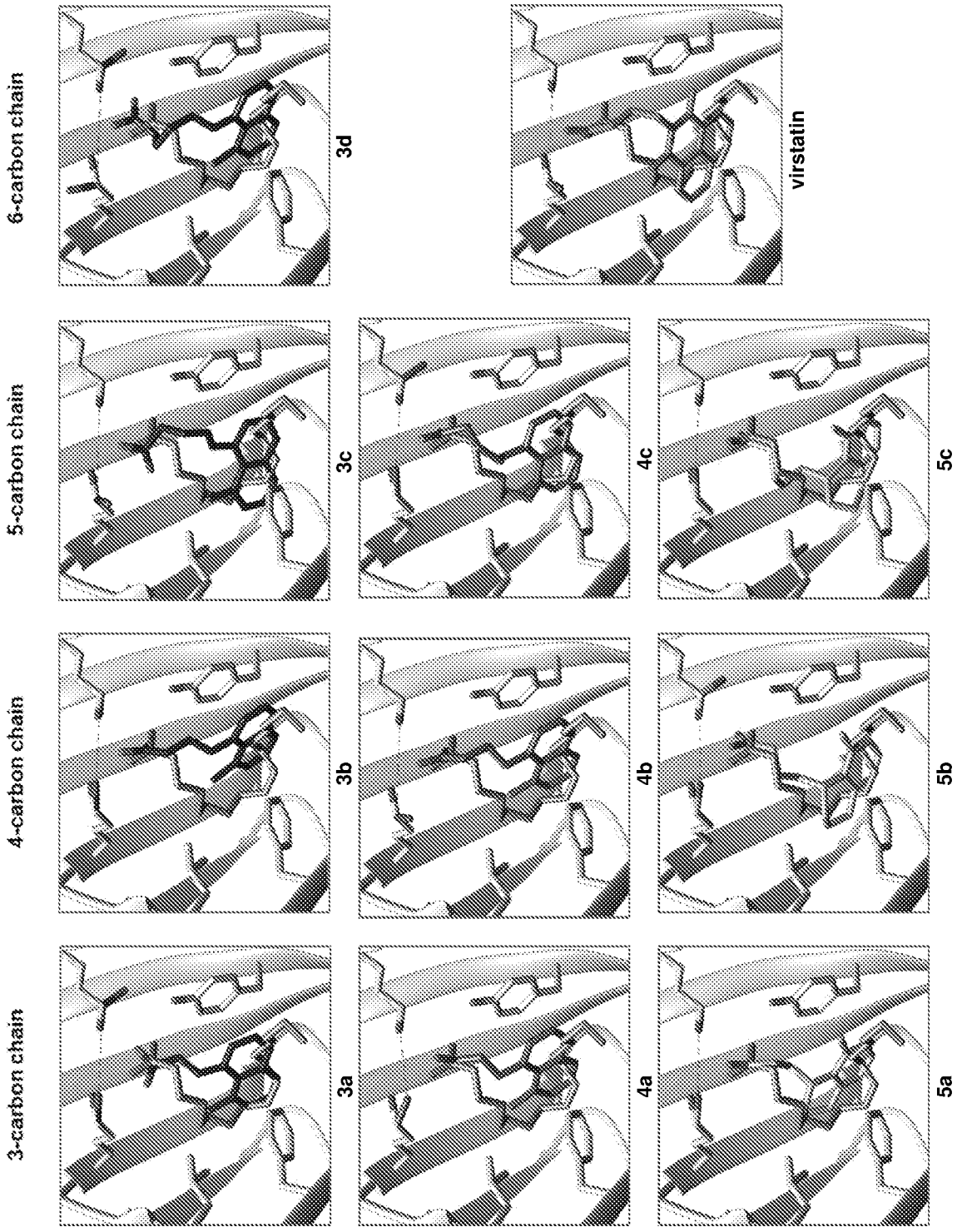
3-carbon chain
4-carbon chain
5-carbon chain
6-carbon chain
Naphthalene, unsaturated side chain
Naphthalene, saturated side chain
Tetralin
3a
3b
3c
3d
4a
4b
4c
virstatin
5a
5b
5c

FIG. 10

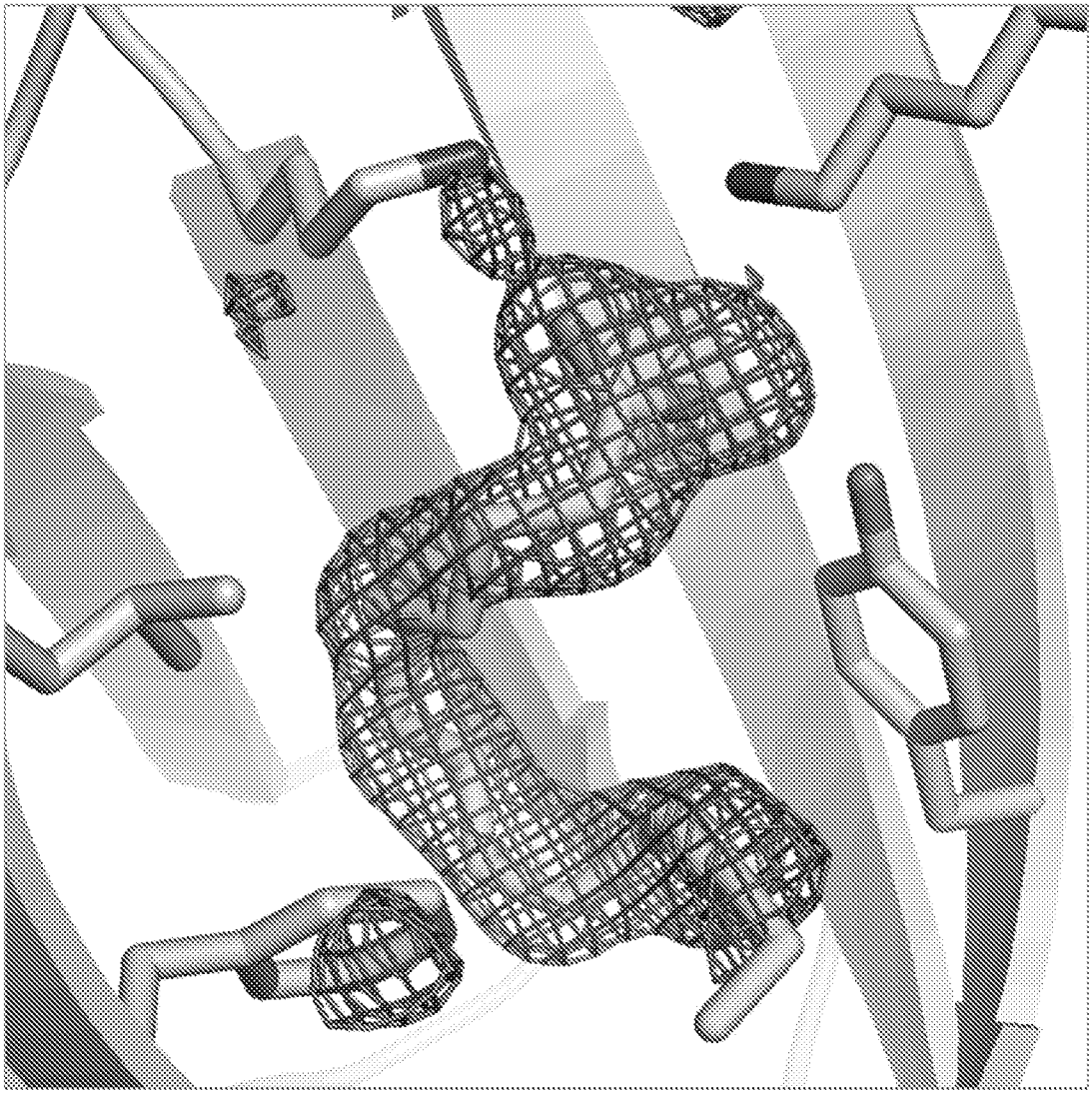

FIG. 11

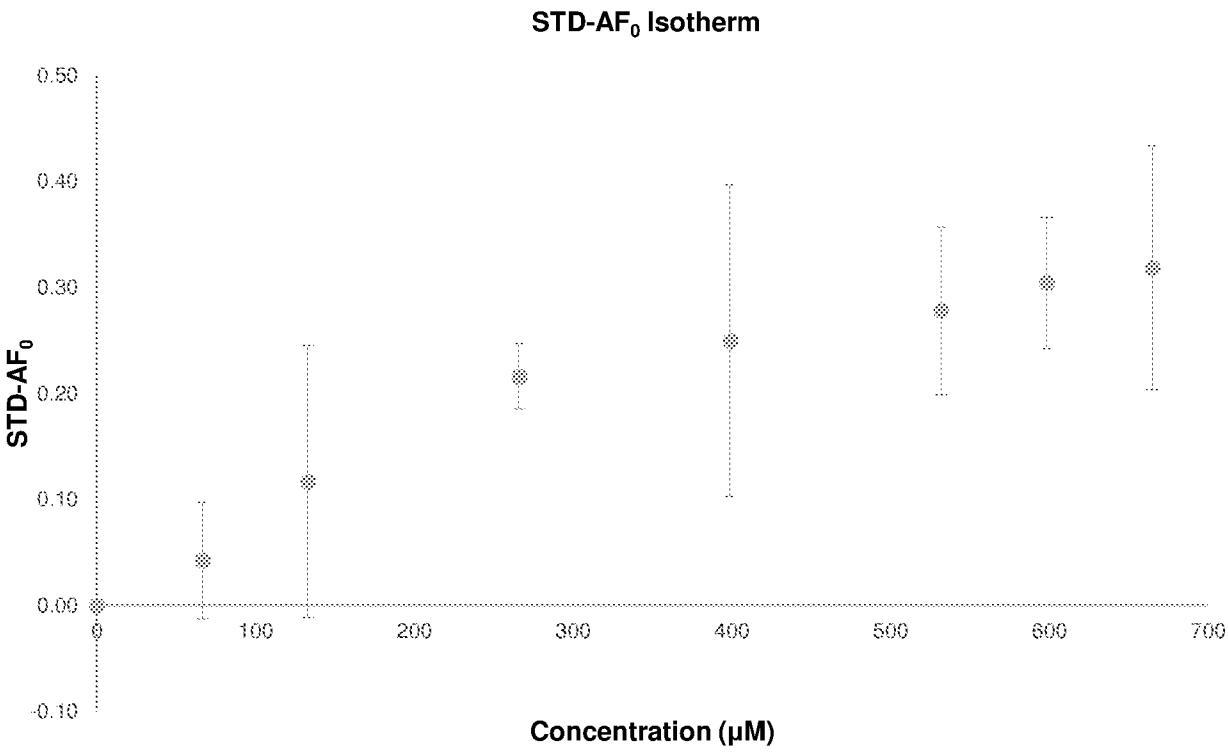
STD-AF0 Isotherm
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
STD-AF0
0
100
200
300
400
500
600
700
Concentration (μM)

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2016/048517

A. CLASSIFICATION OF SUBJECT MATTER (see extra sheet)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07D 209/18, 215/14, 217/16, C07C 57/50 , 57/40, A61K 31/405, 31/47, 31/192, A61P 31/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPTO, DEPATIS, EAPO, PAJ, Espasenet, CAS on STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORSHED M.H et al. «Bacteria Killing Kinetics of the Four Plant Hormones». Pakistan Journal of Biological Sciences, 2005, 8(7), pp. 1025-1029, especially abstract, tables 1- 2, p. 1028, col.1, paragraphs 3, 4	1-22
X	US 3251738 A (GEORGE H. SCHERR) 17.05.1966, tables 1, 2, 3, 5	1-22
X	Database CA [online] GESKE, GRANT D. et al. «Modulation of bacterial quorum sensing with synthetic ligands: Systematic evaluation of N-acylated homoserine lactones in multiple species and new insights into their mechanisms of action.» Journal of the American Chemical Society, 2007, 129(44): 13613-13625, RN 87-51-4, RN 133-32-4, RN 830-96-6 (abstract). Retrieved from STN, AN 148:27358	1, 2
X	Database CA [online] WO 2006/090817 A1 (ONO PHARMACEUTICAL CO) 31.08.2006, RN 89245-41-0 (abstract). Retrieved from STN	1
X	Database CA [online] WO 2005/039546 A2 (VEIJLEN N V) 06.05.2005, RN 17897-49-3, RN 56395-08-5 (abstract) Retrieved from STN, AN 142: 441900	1

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 November 2016 (10.11.2016)	08 December 2016 (08.12.2016)

Name and mailing address of the ISA/RU:
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2016/048517

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database CA [online] US 2004/0186292 A1 (WANG TAO) 23.09.2004, RN 1477-49-2 (abstract) Retrived from STN, AN 140: 303654	1
X	Database CA [online] WO 2007/000436 A1 (JANSSEN PHARMACEUTICA N.V.) 04.01.2007, RN 3243-42-3 (abstract) Retrieved from STN, AN 146:121844	1, 2
X	Database CA [online] EP 737671 A2 (TAKEDA CHEMICAL INDUSTRIES, LTD) 16.10.1996, RN 183962-17-6, (abstract) Retrieved from STN, AN 126:7829	1, 2
X	Database CA [online] BEER J. A. et al. «Chemistry of bacteria. VIII. Synthesis of violacein and related compounds. Ballantine». Journal of the Chemical Society, 1960, 2292-2299, RN 835-45-0, (abstract) Retrieved from STN, AN 54:118162	1, 2
X	Database CA [online] CN 1478761 A (FAMING ZHUANLI SHENQING GONGKAI SHUOMINGSHU) 03.03.2004, RN 22504-86-5, (abstract) Retrieved from STN, AN 142:113345	1, 2
X	DATABASE REGISTRY [online] RN 1096948-84-3, 28.01.2009 Retrieved from STN	1-4, 11
X	DATABASE REGISTRY [online] RN 1094654-43-9, 21.01.2009 Retrieved from STN	1-4, 11
X	DATABASE REGISTRY [online] RN 1082847-53-7, 10.12.2008 Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 1018635-87-4, 01.05.2008 Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 1018591-66-6, 30.04.2008, Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 1018591-62-2, 30.04.2008 Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 1018591-54-2, 30.04.2008 Retrieved from STN	1-4
X	DATABASE REGISTRY [online] RN 1018591-54-2, 30.04.2008 Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 606493-44-1, 19.10.2003 Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 606492-36-8, 19.10.2003, Retrieved from STN	1
X	DATABASE REGISTRY [online] RN 70067-71-9, 16.11.1984 Retrieved from STN	1, 8
X	DATABASE REGISTRY [online] RN 6342-89-8, 16.11.1984 Retrieved from STN	1, 8
X	DATABASE REGISTRY [online] RN 64766-86-5, 16.11.1984 Retrieved from STN	1, 10

C07D 209/18 *(2006.01)*
C07D 215/14 *(2006.01)*
C07D 217/16 *(2006.01)*
C07C 57/50 *(2006.01)*
C07C 57/40 *(2006.01)*
A61K 31/405 *(2006.01)*
A61K 31/47 *(2006.01)*
A61K 31/192 *(2006.01)*
A61P 31/04 *(2006.01)*